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Antibiotic resistance and virulence plasmids in *Campylobacter jejuni*

By

Dobryan M. Tracz ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In

Bacteriology

Department of Medical Microbiology and Immunology

Edmonton, Alberta

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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Antibiotic Resistance and Virulence Plasmids in *Campylobacter jejuni* submitted by Dobryan M. Tracz in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology.

Dr. Mark Peppler

August 25, 2003

Abstract

Campylobacter jejuni is a major food-borne pathogen and a leading cause of bacterial gastroenteritis. Analysis of 203 human clinical isolates of *C. jejuni* (1999-2002) determined 50% of isolates were tetracycline-resistant. Some isolates exhibited extremely high levels of tetracycline resistance (MIC=512 μg/mL, *n*=4). Plasmids were present in 67% of tetracycline-resistant isolates and all contained the *tet*(*O*) gene. Cloning and DNA sequence analysis determined that *tet*(*O*) genes from the 512 μg/mL MIC level have several nucleotide substitutions, but the molecular basis for high levels of resistance has not yet been resolved.

The pVir virulence plasmid was identified in 15% *C. jejuni* clinical isolates through DNA-DNA hybridizations. Incidence of pVir was compared with patient clinical data and was found to be associated with the presence of blood in patient stool, and consequently indicated an invasive infection. This association suggests that pVir may be clinically relevant in *C. jejuni* infections.

"Every little thing is gonna be alright" ***Bob Marley***

"All of us are better when we are loved" ***Alistair McLeod***

Для Мами

For Mama

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Abbreviations

aa-tRNA	Aminoacyl tRNA
Amp	Ampicillin
APLPH	Alberta Provincial Laboratory for Public Health
bp	Base pairs
BFP	Bundle-forming pili
BGOSHU	Bruce-Grey-Owen Sound Health Unit (BGOSHU)
BHI	Brain Heart Infusion
CDT	Cytolethal distending toxin
CFU	Colony forming units
Cm	Chloramphenicol
Cp	Ciprofloxacin
C-terminal	Carboxy terminal
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EF-G	Elongation factor G
EF-Tu	Elongation factor Tu
EPEC	Enteropathogenic *Escherichia coli*
Ery	Erythromycin
GTP	Guanosine 5' triphosphate
HGT	Horizontal gene transfer
IPTG	Isopropylthiogalactoside

Km	Kanamycin
kb	Kilobase
kDa	Kilodaltons
LB	Luria-Bertani
mRNA	Messenger RNA
MF	Microfilaments
MIC	Minimum inhibitory concentration
MT	Microtubuoles
MCS	Multiple cloning site
Nal	Nalidixic acid
NARMS	National Antimicrobial Resistance Monitoring System
NCCLS	National Committee for Clinical Laboratory Standards
N-terminal	Amino terminal
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RBS	Ribosome binding site
RNA	Ribonucleic acid
RPP	Ribosomal protection protein
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
T3SS	Type III secretion system
T4SS	Type IV secretion system

Tc	Tetracycline
tRNA	Transfer RNA
USDA	United States Department of Agriculture
UV	Ultraviolet
UK	United Kingdom

Chapter 1

Campylobacter jejuni infections: Plasmids in antibiotic resistance and virulence

1 *Campylobacter jejuni* infections: Plasmids in antibiotic resistance and virulence

Food-borne infectious diseases are a major cause of human morbidity and mortality worldwide. Clinical symptoms of these infections can range from mild diarrheal diseases to fatal neurological or renal syndromes. A broad spectrum of agents can cause food-borne illness, including bacteria, viruses, parasites, fungi, toxins and prions (99). Bacterial food-borne pathogens are common worldwide and cause substantial impacts on human health. One of the leading bacterial food-borne pathogens is *Campylobacter jejuni*, which is transmitted primarily through contaminated poultry (26). For patients infected with *C. jejuni*, the clinical outcome is dependent on both the susceptibility of the host and the virulence of the pathogen (61).

This thesis considers the importance of plasmids in antibiotic resistance and virulence in *C. jejuni* clinical infections. Surveillance data on antibiotic resistance rates and virulence plasmid incidence will contribute to the clinical understanding and treatment of infection with *C. jejuni*, which is a leading cause of bacterial gastroenteritis. Currently, the mechanisms of *C. jejuni* pathogenesis are poorly defined (162). Insight into the pathogenic mechanisms utilized by *C. jejuni* will assist in the development of successful prevention and control strategies in the management of this significant public health problem.

The scope of this thesis is broad, ranging from the molecular biology of plasmids to the clinical manifestations of *C. jejuni* infections. In this first chapter, the importance of *C. jejuni* as a food-borne infectious disease is reviewed, including an analysis of transmission, clinical symptoms and complications, antibiotic resistance rates and plasmid content. Current knowledge on the pathogenesis of *C. jejuni* and the role of plasmids is also reviewed. All of these topics are directly relevant to the discussion on the role of plasmids in antibiotic resistance and virulence in the outcome of clinical infections with *C. jejuni*. Furthermore, it is important throughout this thesis to consider antibiotic resistance and virulence with an integrated view, as these traits in *C. jejuni* and other bacterial pathogens may occur, and be selected for, in concert.

1.1 Bacterial food-borne infectious diseases

Bacterial food-borne infectious diseases are a significant threat to public health throughout the world. Analysis of data from the Food-borne Diseases Active Surveillance Network (FoodNet) estimates over 5.2 million cases of bacterial food-borne disease occur annually in the United States (99). Several pathogens account for the majority of the cases of bacterial food-borne disease, including infection by *C. jejuni*, *Escherichia coli*, non-typhoidal *Salmonella*, *Listeria monocytogenes*, and intoxication by *Staphylococcus aureus* and *Clostridium perfringens* (Table 1.1). The economic cost of the human illness caused by these bacterial pathogens is substantial, as estimates by the United States Department of Agriculture (USDA) attribute losses of up to US$6.7 billion to food-borne bacterial disease annually (16). Furthermore, although only 5,000 deaths are believed to be caused by food-borne disease annually in the

Table 1.1. Estimated annual cases of bacterial food-borne infectious disease in the United States of America.[a]

Pathogen	Total cases	% of total food-borne cases	Deaths	Hospitilizations
Campylobacter sp.	1,963,141	14.2	99	10,539
Non-typhoidal *Salmonella*	1,341,873	9.7	553	15,608
Escherichia coli (All types)	173,107	1.3	78	2785
Listeria monocytogenes	2,493	0	499	2298
Staphylococcus food poisoning	185,060	1.3	2	1753
Clostridium perfringes	248,520	1.8	7	41
Bacterial subtotal	**4,175,555**	**30.2**	**1297**	**36,466**
Norwalk-like viruses	9,200,000	66.6	310	20,000

[a] Source: (99)

US, bacterial pathogens account for 72% of fatal infections (99).

In the developed world, *C. jejuni* accounts for approximately 50% of total infections caused by food-borne bacterial pathogens annually (99). This exceeds the combined total of estimated *E. coli*, nontyphoidal *Salmonella*, *Shigella* spp. and *Yersinia enterocolitica* infections. In Canada, approximately 12,000 cases of *C. jejuni* gastroenteritis occur annually (56). An estimated 2.4 million infections with *C. jejuni* occur annually in the US, 1.9 million of which are known to be food-borne - the highest of any bacterial food-borne pathogen. In contrast, infections caused by the Norwalk-like viruses are believed to cause over 9 million illnesses (Table 1.1). Under-reporting of these and other food-borne infections have complicated surveillance programs, and the actual figures may be significantly higher. It is important to note that the causes of the large majority of food-borne illnesses are unaccounted for and may be due to novel pathogens (141).

1.2 C. jejuni: The bacterium and its epidemiology

1.2.1 Recognition as a intestinal pathogen

Campylobacters are spiral or curved Gram-negative bacterial rods that are highly motile due to the presence of one or more flagella. Although the importance of *C. jejuni* as a food-borne pathogen is now widely acknowledged, it was only in the 1970's that it became recognized as a major cause of bacterial gastroenteritis because of improved isolation methods and selective media (116). This lag in identification was due to the dependence of *C. jejuni* on strict environmental and nutritional requirements for growth. As a microaerophilic bacterium, *C. jejuni* requires O_2 and CO_2 concentrations of 3-15% and 3-5%, respectively (162). Furthermore, selection of *C. jejuni* is achieved by growth on complex medium at 42°C. The ability of *C. jejuni* to grow at 42°C is believed to be the result of adaptation to the intestinal tract of its natural host, birds (26, 65).

1.2.2 Avian carriage of *C. jejuni* and the importance of poultry

As a commensal of the avian intestinal tract, *C. jejuni* can be shed into the environment by migrating birds, which are considered a major reservoir for *C. jejuni* in the environment (65). Although this can lead to the contamination of coastal waters and wetlands, the impact on *C. jejuni* epidemiology is believed to be minimal. This conclusion is the result of studies on wild birds like the black-headed gull, a likely avian candidate for transmission of enteric pathogens (14). Strains of *C. jejuni* isolated from human patients were distinct from the gull isolates, and although they may be a reservoir, gulls are more likely to only be an accidental vehicle for human *C. jejuni* infections (14). Another *Campylobacter* species, *Campylobacter lari*, is also commonly transmitted by gulls (79).

The primary source of *C. jejuni* infection in humans occurs through consumption or contact with raw or under-cooked poultry, including cross-contaminated food products (26). Various types of poultry have been found to contain extremely high numbers of campylobacters, both throughout and subsequent to the slaughtering process. Studies from the Europe and North America show over 85% of raw chickens sold in supermarkets are contaminated with *Campylobacter* spp (Table 1.2). In agricultural operations, flocks of chicken are rapidly colonized by *C. jejuni* within weeks of birth. High population levels of *C. jejuni* (10^5-10^9 colony forming units (CFU)/gram) are present in chicken intestinal tracts (8, 26). Carriage continues throughout the bird's lifetime and shedding of campylobacters occurs intermittently. In contrast to *Salmonella* sp., another commensal of poultry, transmission of *C. jejuni* is not believed to penetrate chicken egg shells (26). An excellent review on controlling *C. jejuni* contamination in poultry products is available (26). *C. jejuni* is also a part of the normal intestinal flora of a number of agricultural animals, such as cattle, which may contribute to food-borne transmission to humans through milk or meat products.

1.2.3 Epidemiology

When the ubiquitous nature of *C. jejuni* in poultry is combined with a low infectious dose of 500 CFU (122), the potential for transmission is significant for humans in contact with poultry products. However, the infectious dose can vary depending on a person's diet. Human infections with *C. jejuni* are primarily sporadic, resulting from point source ingestion of contaminated food products. Transmission from person to person is not common, but can occur through the fecal-oral route, especially with young children. Furthermore, transmission

Table 1.2. Incidence of *C. jejuni* in poultry products.[a]

Poultry source	Country	Content	Reference
Live birds			
	Japan	20%	(21)
	Ireland	90%	(69)
	England	94%	(166)
Chicken carcasses			
	Canada	94%	(77)
	Denmark	87%	(53)
	Belgium	85%	(160)

[a] Based largely upon references collected by (26)

can occur from house pets such as puppies or kittens. Large community outbreaks are rare, but can occur when there is ingestion of unpasteurized milk (76) and contaminated water among the public (101). The latter is exemplified by the outbreak of diarrheal illness in Walkerton, Ontario, Canada in 2000, in which *C. jejuni* was identified along with *E. coli* O157:H7 in patients (159).

In their investigative report, the Bruce-Grey-Owen Sound Health Unit (BGOSHU) determined that over 2300 people in Walkerton, Ontario became ill from the consumption of water contaminated with fecal run-off from nearby farmland. Although it was not believed to be responsible for any of the seven deaths in this outbreak, *C. jejuni* was isolated from patient stool samples at levels just below *E. coli* O157 (8% and 13%, respectively) (159). In the Walkerton outbreak, patients infected with *C. jejuni* reported symptoms similar to those infected with *E. coli* O157, except for higher rates of bloody diarrhea in *E. coli* cases. Thus, the presumed presence of *C. jejuni* in water supplies could be the source of outbreaks of gastroenteritis and could serve as an indicator of fecal contamination (65), although isolation of *Campylobacter* sp. from water is difficult, as none were isolated in the Walkerton outbreak.

1.3 Clinical manifestations of infection with C. jejuni

C. jejuni is the primary human pathogen among *Campylobacter* sp. (68), and along with *Campylobacter coli*, accounts for over 95% of infections (79). Occasionally other species, such as *Campylobacter fetus* or *Campylobacter* *upsaliensis* (79) can also cause gastroenteritis in humans. Primarily a commensal of pigs, *C. coli* causes a human diarrheal disease similar to that of *C. jejuni*, but on a less severe scale (133). *C. fetus* also causes gastroenteritis in humans, but is commonly associated with spontaneous abortions in cattle (79).

Clinically, infection with *C. jejuni* in developed countries frequently causes an acute diarrheal disease, in which the patient suffers from intense abdominal pain, diarrhea and fever (Table 1.3) (11). Other symptoms may include nausea, vomiting, headache and the presence of mucous and blood in the feces, the latter indicating a particularly severe invasive infection (133). After an incubation period of one to seven days, an initial stage of abrupt cramping leads into a profuse diarrheal stage in which ten bowel movements per day is not uncommon (133). Morbidity is difficult to measure, but is approximately five days, although the patient may continue to shed *C. jejuni* in their feces for several weeks after recovery. Dehydration may lead to hospitalization in 5 to 10% of infected individuals who usually recover completely assisted by some protective immunity (133). Mortality rates are low and occur primarily in the young, elderly, immuno-suppressed individuals, or those that have an underlying disease.

In addition to gastrointestinal diseases, complications can occur during *C. jejuni* infections (Table 1.4), such as the development of Guillian-Barré syndrome, a neurological disorder that results in acute flaccid paralysis (52). Current estimates suggest one in 1000 infections with *C. jejuni* lead to Guillian-

Table 1.3. Clinical symptoms associated with infection with *C. jejuni*. Frequencies are calculated from *C. jejuni* gastroenteritis outbreaks involving 25 or more individuals.[a]

Symptom	**Frequency**
Diarrhea	84%
Abdominal Pain	79%
Fever	50%
Headache	41%
Vomiting	15%
Blood in stool	15%

[a] Source: (133)

Table 1.4. Diseases associated with *C. jejuni* infection.[a]

Gastrointestinal
Enteritis
Toxic megacolon
Pancreatitis
Gastic ulceration
Splenic rupture

Rheumatological
Reactive arthritis
Reiters syndrome

Neurological
Guillian Barre Syndrome
Meningitis

Skin
Cellulitis
Abscess

Lung
Pneumonia
Empyema

Intravascular
Bacteremia
Septic shock
Hemolytic Uremic Syndrome
Aneurysm

[a] Source: (27)

Barré syndrome (106). Furthermore, in rare cases *C. jejuni* may enter the bloodstream and lead to bacteremia (117, 133). Other long term sequelae include reactive arthritis and Reiter's syndrome. The clinical outcome of *C. jejuni* infection is dependent on the virulence of the infecting strain, but the susceptibility of the host is also a major factor (133), especially with prolonged or complicated illness (18).

1.4 Treatment

C. jejuni gastroenteritis may be an uncomfortable disease to acquire, but it is rarely fatal and is primarily a self-limiting infection. For these reasons, clinical treatment consists principally of oral replacement of fluid and electrolytes lost through vomiting and diarrhea. Antibiotic treatment is reserved for clinical cases in which the patient is acutely ill with a severe or complicated infection, in systemic infections of immuno-suppressed individuals and for control of infections in high risk groups (133). When antibiotic treatment is administered, erythromycin has traditionally been the drug of choice and continues to be used effectively in clinical treatment of *C. jejuni* enteritis (108). Ciprofloxacin may also be used for prophylaxis of traveller's diarrhea or for treatment of enteric infections. However, the use of these antibiotics in some countries is under threat of the emergence of drug-resistant strains of *C. jejuni*.

1.5 *Antibiotics: Modes of action and mechanisms of resistance*

Antibiotics are small, low-weight molecular compounds that are used to kill bacteria or inhibit their growth. Generally, antibiotics have specific targets in the bacterial cell, acting to inhibit processes like protein synthesis, DNA replication and cell wall synthesis. In treatment of *C. jejuni* gastroenteritis, erythromycin is a protein synthesis inhibitor and ciprofloxacin targets DNA replication. Tetracycline, an antibiotic to which there is now substantial resistance in *C. jejuni*, is an inhibitor of protein synthesis.

There are four main mechanisms of resistance to antibiotics: (1) efflux of the antibiotic from the host cell, (2) modification or destruction of the antibiotic, (3) alteration of the antibiotic target site or (4) protection of the antibiotic target site (28). In *C. jejuni*, mutation of the target site is important in resistance to ciprofloxacin and erythromycin, whereas protection of the target is important in resistance to tetracycline.

1.5.1 Fluoroquinolones: Ciprofloxacin

Fluoroquinolones are a chemically modified form of the quinolone antibiotic, nalidixic acid. They target two bacterial enzymes important in DNA replication, DNA gyrase (type II topoisomerase) and DNA topoisomerase IV. DNA gyrase introduces negative supercoils in the DNA and topoisomerase IV is important in unlinking activity during DNA replication (31). As both enzymes are essential, the inhibition of their action results in cessation of bacterial growth. Fluoroquinolones trap these enzymes into a complex, prevent DNA replication and effectively poison the cell (31). The major antibiotic in this class is ciprofloxacin, which is used to treat a wide variety of infections, such as traveller's diarrhea caused by enteric pathogens, including *C. jejuni*.

1.5.1.1 Fluoroquinolone resistance – a historical background

Upon their introduction in the 1980's, fluoroquinolones were greeted with widespread enthusiasm from the medical community for treatment of bacterial infections. Their good pharmacological properties contributed to their clinical efficacy in treating gastroenteritis caused by *C. jejuni* and other enteric pathogens (107). Although fluoroquinolones were successful in initial clinical trials for treating patients with gastroenteritis caused by *C. jejuni* (176), resistance quickly emerged.

A study from the Netherlands was the first to demonstrate that the emergence of fluoroquinolone resistance among human clinical isolates of *C. jejuni* had a temporal relationship with the licensing of fluoroquinolones for use in the animal agriculture industry (33). In 1987, the fluoroquinolone enrofloxacin was introduced in the Netherlands for treatment of infections in poultry. Resistance profiles of both human and chicken *C. jejuni* isolates prior to the use of enrofloxacin (1982-1985) showed no resistance to ciprofloxacin. In the years after the introduction of enrofloxacin, frequencies of ciprofloxacin resistance had increased to 11% and 14% for humans and chickens, respectively (33). Further evidence for the link between fluoroquinolone resistance in poultry products and human infections came from studies in the United States, in which molecular subtyping demonstrated ciprofloxacin-resistant *C. jejuni* from human clinical infections and locally purchased retail poultry products were virtually identical (134, 135).

1.5.1.2 Mechanisms of fluoroquinolone resistance

Resistance to fluoroquinolones is primarily through a mutation in the DNA sequence encoding the *gyrA* subunit of the DNA gyrase enzyme (31). A single point mutation at Thr-86, Asp-90 or Ala-70 in *gyrA* can result in resistance. Higher-level fluoroquinolone resistance is associated with mutation at the Thr-86 position, which is also the most common mutation site (167). Mutants resistant to nalidixic acid arise at a frequency of 10^{-8} in *C. jejuni* and are cross-resistant to fluoroquinolones (151). These mutations can be induced by treating poultry flocks with fluoroquinolones, as McDermott *et al*. (97) found that fluoroquinolones did not eliminate *C. jejuni* from the intestinal tracts of chickens, but rapidly selected for resistant mutants.

Efflux of ciprofloxacin may be of importance in fluoroquinolone resistance in *C. jejuni*. The *cmeABC* gene operon encodes a multi-drug efflux pump that contributes to intrinsic resistance of *C. jejuni* to a number of antibiotics (88). The CmeABC efflux system is structurally and functionally similar to that of efflux systems in other Gram-negative bacterial pathogens. Disruption of the CmeABC mediated-efflux results in intracellular accumulation of ciprofloxacin and increases susceptibility to various other antimicrobial agents (88). However, recent research suggests efflux likely plays only a minor role in fluoroquinolone resistance in *C. jejuni* (114).

1.5.2 Macrolides: Erythromycin

The macrolides, such as erythromycin, are considered to be clinically safe antibiotics that are effective against *C. jejuni* gastroenteritis. Erythromycin is a bacteriostatic agent that acts as a protein synthesis inhibitor. It binds reversibly to the 50S subunit of the 70S bacterial ribosome and causes dissociation of the peptidyl-tRNA, which halts the elongation cycle of protein synthesis at the transpeptidation step (109, 158).

1.5.2.1 Mechanisms of erythromycin resistance

Levels of resistance to erythromycin, the primary drug of choice in treating *C. jejuni* gastroenteritis, are low in Canada (43). Erythromycin resistance cannot be selected for *in vitro* and few studies of the mechanism have been undertaken. Several mechanisms of macrolide resistance are known to occur in other bacteria, including *erm*-mediated modification of the target site, antibiotic efflux and *ere*-mediated inactivation of the antibiotic (109). In *C. jejuni*, resistance to erythromycin is most likely due to the alteration of the ribosome target site on the 23S rRNA. These genes have been sequenced in erythromycin-resistant *Campylobacter* spp., and the mutation of an adenine residue in the 23S rRNA has been identified (158), but the type of base substitution is disputed (63). A study by Yan and Taylor (178) has ruled out the possibility of efflux, antibiotic modification or the action of an RNA methylase.

The mechanisms of erythromycin resistance in *C. jejuni* and *C. coli* have been reviewed (142, 158).

1.5.3 Tetracycline

The tetracyclines are a group of broad spectrum antibiotics that have been used against a wide variety of human pathogens. Chlorotetracycline was the first to be discovered in 1948, followed by both oxytetracycline and tetracycline in 1953 (19). Like many antibiotics, they were isolated from a soil microorganism, *Streptomyces* sp., and have since been chemically modified to improve their clinical properties. These semi-synthetic members of the tetracycline family include doxycycline, an important antibiotic in the clinical treatment of sexually transmitted diseases, and glycylcycline, the newest tetracycline.

Like the macrolides, the tetracyclines are protein synthesis inhibitors which act at the level of the bacterial ribosome. Initially, tetracycline is accumulated in the bacterial cell by active uptake. Once inside the bacterium, tetracycline binds reversibly to a single high-affinity site on the 30S subunit of the 70S ribosome, as well as several other low-affinity sites (19). Binding to the high-affinity site prevents association of the aminoacyl-tRNA (aa-tRNA) with the A site on the ribosome. This stops the elongation step of protein synthesis, in which the aa-tRNA normally decodes the mRNA transcript and delivers the amino acid to the growing polypeptide chain.

1.5.3.1 Tetracycline resistance

Resistance to tetracycline is mediated by a number of *tet* determinants that can transfer between bacterial genera, including genes for efflux and ribosomal protection (see: http://faculty.washington.edu/marilynr). The mechanism of tetracycline efflux involves membrane proteins such as Tet(A) that actively pump out tetracycline from the bacterial cell. The tetracycline efflux proteins are the best studied of the tetracycline resistance proteins and in Gram-negative bacteria, efflux genes are commonly carried on plasmids (19). However, the mechanism of tetracycline resistance in *C. jejuni* is through ribosomal protection, which involves a soluble Tet(O) protein that induces a conformational change in the ribosome, which ejects tetracycline from the ribosome and allows protein synthesis to occur (23).

1.5.3.2 Tet(O) and ribosomal protection

In *C. jejuni*, tetracycline resistance is primarily mediated by transmissible plasmids (146), harbouring the *tet*(*O*) gene (148). Originally, cloning and sequencing of *tet*(*O*) (GenBank Accession Number M18896) identified a gene of 1917 base pairs (bp) (93), but recent analysis has corrected the 3' end of the *tet*(*O*) sequence, which is now known to be 1920 bp (22). The G+C% content of *tet*(*O*) is 40% (93), much higher than that of the *C. jejuni* genome (30%) (113), leading to speculation that *tet*(*O*) had its origin in the Gram-positive bacteria (136). Originally cloned from the plasmid pUA466 (148), *tet*(*O*) was found to mediate tetracycline resistance up to a minimum inhibitory concentration (MIC) of 64 μg/mL of tetracycline in both *C. jejuni* and *E. coli* JM107 (143). Furthermore, it is believed that the upstream region of *tet*(*O*), which contains two putative promoters (P1 and P2), is important for full expression of tetracycline resistance (169).

Encoded by the *tet*(*O*) gene is the 640 amino acid (~72 kDa) protein, Tet(O), a ribosomal protection protein (RPP) that confers resistance by actively displacing tetracycline from its primary binding site (24, 157). This soluble cytoplasmic protein is similar to other members of the RPP group, including Tet(M), Tet(P), Tet(Q), Tet(S), Tet(T), Tet(W) and Otr(A) (158). Of the ribosomal protection proteins involved in tetracycline resistance, Tet(O) and Tet(M) are the best characterized (19), which share approximately 76% sequence similarity (93). Tet(M) is the most widely distributed RPP and is known to be present in eight Gram-negative bacterial genera and 18 Gram positive genera (19). For example, 79% of tetracycline-resistant oral bacteria contain *tet*(*M*) (165). It is noteworthy that no RPPs have been identified in the *Enterobacteriaceae*.

Tet(O) has substantial sequence similarity to the ribosomal elongation factors EF-Tu and EF-G, which are essential in bacterial protein synthesis. Specifically, the N-terminal region of Tet(O) has the highest amino acid identity and is known to possess GTPase activity (149). The elongation factors interact with the ribosome in a transient manner during the process of protein synthesis. EF-G assists in translocation of the aa-tRNA from the ribosomal A site to the P site. Therefore, Tet(O) may act as a molecular mimic of EF-G and EF-Tu that binds to and causes a conformational change in the ribosome, promoting the release of tetracycline (23).

A DNA probe has been used successfully to identify *tet*(*O*) in plasmids and chromosomal DNA from *C. jejuni* (81). DNA from virtually all tetracycline-resistant isolates from both human and chicken sources hybridized with the *tet*(*O*) probe. Human clinical isolates of *C. jejuni* had comparable *tet*(*O*) content on their plasmids (47%) and chromosomal DNA (44%), while the majority of *C. jejuni* isolates from chickens carried *tet*(*O*) on plasmids (87%) (81). Therefore, plasmids play a major role in the dissemination of tetracycline resistance in *C. jejuni*.

The widespread prevalence of tetracycline resistance in *C. jejuni* human clinical isolates is believed to be due to the use of tetracycline in veterinary medicine for the control of infections and at sub-therapeutic levels for growth promotion (82, 98). This effect is similar to that of fluoroquinolone use in animal agriculture. Research by Stuart Levy in the 1970's provided strong evidence that the inclusion of tetracycline in animal feed leads to emergence of tetracycline-resistant *E. coli* in the intestinal flora of humans (85, 86). Resistant bacterial strains are selected for in the treated animal population and transmitted to humans through meat products or alternate routes, such as food in contact with water contaminated by animal feces (177).

1.5.4 Resistance to other antibiotics

Resistance frequencies to chloramphenicol, a protein synthesis inhibitor, are low in *C. jejuni*. The mechanism of chloramphenicol resistance is through the action of the chloramphenicol acetyl transferase (CAT) enzyme, which modifies the antibiotic and renders it ineffective (168). Resistance to the aminoglycosides, such as kanamycin, is also rare in *C. jejuni* and involves the modification of the antibiotic by an aminoglycoside phosphotransferase (APH) (158). These resistance determinants are normally carried on plasmids (153).

1.6 *Frequency of antibiotic resistance in* C. jejuni

In *C. jejuni*, antibiotic resistance has major clinical implications. Erythromycin is the first line drug in the treatment in severe infections and ciprofloxacin is commonly used to treat various types of bacterial gastroenteritis. The emergence of multi-drug resistance is especially problematic, since it effectively limits the availability of effective antibiotic chemotherapy. Current worldwide antibiotic resistance frequencies in *C. jejuni* show a diverse response to different antibiotics. However, there are global trends in the increasing incidence of resistance to several antibiotics, such as tetracycline. In Alberta, antibiotic-resistance frequencies in *C. jejuni* have not been determined since the early 1980's (145).

1.6.1 Frequency of resistance to fluoroquinolones

Although fluoroquinolones initially had excellent clinical efficacy, the 1990's showed an extraordinary rise in the incidence of ciprofloxacin resistance in *C. jejuni*. Excellent data on the emergence of fluoroquinolone resistance comes from Spain, where levels were 0-2% before 1989 and 88% in 1996 (120, 123). Studies on resistance in Ontario and Quebec, Canada have found levels increased to 13% in 1995, up from 0% before 1989 (44, 67). The Campylobacter Sentinel Surveillance Scheme (CSSS), established in the UK in May, 2000, has found that *Campylobacter* infections acquired abroad were more likely to be resistant to ciprofloxacin than nationally acquired strains (55% to 10%, respectively) (130).

1.6.2 Frequency of resistance to erythromycin

Although antibiotic-resistance frequencies in a number of bacterial pathogens are increasing annually, the frequencies of erythromycin resistance in *C. jejuni* have remained very low worldwide. This is encouraging data for clinicians, who can continue to use erythromycin as the first drug of choice in treating *C. jejuni* gastroenteritis. In Alberta, no erythromycin resistance was observed in 1980-81 (145). Globally, frequencies range from low levels (0%) in Denmark (1), up to 10% in Taiwan (87).

1.6.3 Frequency of resistance to tetracycline

The incidence of tetracycline resistance among human clinical isolates of *C. jejuni* has shown a dramatic upward trend since the 1980's (Table 1.5). In one Canadian study, tetracycline resistance in human clinical isolates of *C. jejuni* increased to 56% from 19% in just over a decade (43). In Alberta,

Table 1.5. Tetracycline resistance (Tc^r) in *Campylobacter* and *C. jejuni* isolated from human and animal sources worldwide.

Year and Country	Rate of Tc^r	Organism	Animal source	Source or reference
1999, United States	85%	*C. jejuni*	Raw retail meats	(46)
1994, Taipei	78%	*C. jejuni*	Humans	(81)
1995-1997, Canada	56%	*C. jejuni*	Humans	(43)
2000, Ireland	31%	*C. jejuni*	Humans	(91)
1997, Northern Ireland	26%	*Campylobacter* spp.	Chickens	(104)
2000, Northern Ireland	19%	*Campylobacter* spp.	Humans	(104)
1980-1983, Canada	15%	*C. jejuni*	Humans	(78)
1997, Denmark	11%	*C. jejuni*	Humans	(1)
1981, Canada	9%	*C. jejuni*	Humans	(145)
1994, India	9%	*C. jejuni*	Humans	(118)
1997, Chile	2%	*C. jejuni*	Humans	(38)

Canada, tetracycline resistance levels were found to be 6.8% and 8.6% in 1980 and 1981, respectively (145).

Globally, there is an increasing trend in the incidence of tetracycline resistance in *C. jejuni*. In Japan and the United States 55% of clinical isolates are tetracycline-resistant (105, 126). The extremely high frequencies of tetracycline resistance among strains of *C. jejuni* have effectively removed tetracycline from the list of antibiotics available to treat *C. jejuni* gastroenteritis in Thailand (34), where up to 95% of isolates are tetracycline-resistant (87). High frequencies of resistance are found among poultry, including one study that found all isolates from chickens were tetracycline-resistant (*n*=167) (81).

1.6.4 Multi-drug resistance

Strains of *C. jejuni* that are resistant to multiple antibiotics pose a major challenge for effective clinical treatment. This is especially serious in areas of the world where antibiotic resistance in enteric pathogens has reached alarming frequencies. In Thailand, where *C. jejuni* is resistant to many antibiotics, there is 100% co-resistance between macrolides (azithromycin) and fluoroquinolones (58). In Taiwan, nalidixic acid-resistant strains were co-resistant to tetracycline and ciprofloxacin at frequencies of 97% and 66%, respectively (87).

1.7 Plasmids in C. jejuni*: Incidence and antibiotic resistance*

Plasmids are extra-chromosomal genetic elements in bacteria that can carry genes for antibiotic resistance and can undergo inter- and intra-species transfer. In *Campylobacter*, plasmid content varies from 13% to 91%, depending on the source of the isolates (Table 1.6). On average, 43% of human clinical isolates of *C. jejuni* contain plasmids and high plasmid content has also been described in animal isolates of both *C. jejuni* and *C. coli*. Isolates with multiple plasmids have been identified in human clinical isolates, and plasmid sizes range from 4.3 to 50 kb (145), with other studies reporting extremely large plasmids (>100 kb) (154).

In animals, a study by Bradbury and Munroe (13) found that there was a higher occurrence of plasmids in *C. jejuni* isolated from cattle with a diarrheal disease than from healthy animals (31.2% vs. 13.6%, respectively). This suggests a relationship between enteric infections in animals and the presence of plasmids in *C. jejuni*. That same study found that in chickens, the primary source of human *C. jejuni* infections, plasmids were found in 75.8% of isolates (13).

Plasmids in *C. jejuni* have previously been found to encode tetracycline resistance, giving an MIC of 32 to 128 μg/mL of tetracycline (145). Conjugative transfer of these plasmids has been demonstrated between *Campylobacter* sp., but not to *E. coli*, suggesting their host range is restricted (146). The pTet tetracycline-resistance plasmid is approximately 37 kb (5), although larger tetracycline resistance plasmids (~100 kb) have been reported (154). Other plasmids isolated from *C. jejuni* include a small plasmid that carriers the *aphA*-7 kanamycin-resistance determinant (153) and a small plasmid (pCJ01) that may be developed for use as an expression vector (92). Overall, plasmids in

Table 1.6. Plasmid content in *Campylobacter* spp., *C. jejuni* and *C. coli*. Animal source of the isolates is noted.

Organism	Source of *Campylobacter*	Plasmid content	Sample Size	Reference
C. jejuni	Chickens	91%	167	(81)
C. jejuni	Chickens	75%	62	(13)
C. coli	Humans	70%	10	(126)
C. coli	Pig	68%	71	(13)
C. coli	Humans, pig, chicken	67%	6	(144)
C. jejuni	Humans	52%	108	(126)
C. coli	Unknown	47%	17	(12)
C. jejuni	Humans	44%	41	(81)
C. jejuni	Humans	35%	282	(154)
Campylobacter sp.	Humans	33%	30	(4)
Campylobacter sp.	Humans	29%	242	(89)
C. jejuni	Chickens, pets	29%	346	(154)
C. coli	Humans	29%	28	(154)
C. jejuni	Unknown	28%	40	(12)
C. jejuni	Humans, cows	25%	8	(144)
C. jejuni	Dairy cows and cattle	24%	54	(13)
Campylobacter sp.	Humans	21%	121	(2)
C. jejuni	Humans	13%	8	(150)
Overall average		**43%**		
***C. jejuni* only**		**42%**		

C. jejuni play a role in the dissemination of tetracycline resistance, and may also play a role in pathogenesis.

1.8 Mechanisms of pathogenicity

Although *C. jejuni* has been recognized as a leading food-borne bacterial pathogen, the mechanisms of its pathogenesis remain poorly understood (162). In contrast, the pathogenesis of other enteric pathogens, such as *Salmonella*, has been well characterized. Enteric pathogens use a number of different strategies to subvert human host defenses and cause infections. However, common themes in pathogenesis have been identified among these enteric pathogens, such as the importance of adherence and invasion of the intestinal epithelium. The virulence of the pathogen is central to the clinical outcome for a patient infected with the bacterium, although the susceptibility of the host is also important. As defined by Casadevall (18), “virulence” will herein refer to the relative capacity of the pathogen to damage the host.

A number of virulence factors have been proposed to contribute to the pathogenesis of *C. jejuni*, which allow for adherence, colonization and invasion of the intestinal epithelium (Table 1.7). Potential virulence components include flagella (for motility and chemotaxis), invasion proteins (for adherence and invasion) and the production of toxins (for the production of diarrhea) (61). Although the genome of *C. jejuni* NCTC 11168 has been sequenced (113), the importance of these virulence factors in the pathogenesis of *Campylobacter* has

Table 1.7. Role of virulence factors in *C. jejuni* pathogenesis.[a]

Virulence property	**Virulence factor**
Motility and chemotaxis	Flagella
Adhesion	CadF, Ped1
Invasion	pVir?
Toxins	Cytolethal distending toxin
Secreted effector proteins	Cia proteins ?

[a] Source: (61)

not been completely resolved (see below). The virulence of strains is important to the discussion of clinical outcomes of infection with *C. jejuni*.

C. jejuni strain 81-176 was originally isolated from an outbreak of *C. jejuni* gastroenteritis from unpasteurized milk (76) and has been well characterized at both the molecular and clinical levels. Studies on secretion of toxins and virulence factors, induction of host cytoskeletal re-arrangements, host cell inflammatory responses, lipooligosaccharide variation and invasion have all been performed with 81-176 (5, 7, 49, 60, 75, 125). An *in vivo* study involving experimental human infections with 81-176 has shown the production of inflammatory diarrhea (9).

1.9 Virulence factors

1.9.1 Motility and chemotaxis

In order to colonize the intestinal epithelium, an enteric pathogen must be able to penetrate the mucosal layer that covers the intestinal cells. The ability to penetrate this mucus layer is largely dependent on the motility of the bacterial pathogen. *C. jejuni* is known to be a highly motile bacterium, due to the possession of one or more polar flagella, and can efficiently move through the mucous barrier (138).

The flagella of *C. jejuni* are composed of flagellin subunits, encoded by the *flaA* and *flaB* genes, which are known to undergo antigenic variation (55). FlaA is the dominant protein subunit in *C. jejuni* flagella, but FlaB is believed to still play an important role in motility (172). Approximately 36 genes may be involved in flagellar biosynthesis, based upon annotation of the *C. jejuni* NCTC 11168 genome (113).

Chemotaxis, or the ability to sense and respond to chemical gradients is an essential factor in *C. jejuni* pathogenesis. *C. jejuni* shows attraction to mucins and L-serine (162). The importance of chemotaxis was demonstrated by Takata *et al.* (140), who showed non-chemotactic mutants were unable to colonize animal intestinal tracts. A key response regulator that may influence *C. jejuni* virulence gene expression is CheY, which has been found to be important in colonization (181). Overall, the ability to sense the mucosal layer and move through it by the force of flagellar motion is a critical element of *C. jejuni* pathogenesis.

1.9.2 The role of toxins

Enteric pathogens are known to secrete protein toxins that induce diarrhea in the human host. The prototype enterotoxin is produced by *Vibrio cholerae*, the classic diarrheagenic pathogen. There have been reports of cholera-like toxin activity in *C. jejuni* (124), but only the cytolethal distending toxin (CDT) has been identified and well characterized (64, 174). In the analysis of the genome sequence of *C. jejuni* NCTC 11168, Parkhill *et al.* (113) could not identify any novel toxin genes, other than the CDT genes and a putative protein with a hemolysin domain. No cholera-like toxin gene has been conclusively identified.

CDT was first identified by Johnson and Lior (64), who demonstrated cultured cell lines (including HeLa cells) would distend and die when exposed to a CDT-producing *C. jejuni* isolate. All *C. jejuni* and related species (*C. coli* and *C. fetus*), as well as some *E. coli* and *Shigella* sp., can produce CDT. Cytotoxicity results when CDT blocks the host cell cycle at the G2 phase (174). The inability of host cells to continue their cell cycle is likely due to the inactivation of CDC2 kinase, which is required for mitosis. CDT can also directly mediate the release of the inflammatory cytokine interleukin-8 (IL-8) from intestinal epithelial cells (57). IL-8 is important for the influx of neutrophils in the primary immune response. The diarrheal symptoms observed in *C. jejuni* clinical infections have been proposed to be the result of CDT affecting the survival of intestinal epithelial cells and their absorptive functions (162), but its actual role in disease is not proven.

1.9.3 Adherence

Bacterial pathogens often utilize cell surface structures like pili or fimbriae to mediate adherence to eukaryotic cells. Adherence to the cells of the host gastrointestinal tract is necessary for enteric pathogens, as peristalsis and fluid flow would otherwise sweep the microorganisms away (72). Thus, adherence to intestinal epithelial cells is critical to *C. jejuni* pathogenesis. This fact is supported by evidence that demonstrates *C. jejuni* strains from patients with more severe symptoms (fever, diarrhea) are more adherent in cell culture assays than strains isolated from patients without overt clinical symptoms of *C. jejuni* gastroenteritis (36).

A number of molecules on the *C. jejuni* cell surface may be important in adhesion. *C. jejuni* may contain fimbrial-like structures, but this has not yet been confirmed (162). No pilus like structures were encoded on the chromosome of *C. jejuni* NCTC 11168 (113). A potential adhesin is PEB1, as *peb1* knockouts exhibited reduced adherence and colonization of HeLa cells (115). Another adhesin, CadF, binds to fibronectin (70) and is important for colonization of chicken intestinal tracts (183). In addition to their importance in motility, flagella also are important in adhesion (180). Adherence is a necessary step for invasion of the intestinal epithelium, but not all strains of *C. jejuni* are capable of invasion.

1.9.4 Invasion

Invasion refers to the process of bacterial internalization into non-phagocytic cells in the host and should not be confused with 'invasive' infections which spread throughout the host (75). Invasive pathogens, such as *Salmonella* and *Shigella*, are well known to hijack the host cell's cytoskeleton and produce major morphological changes or membrane ruffling on the host cell surface (47). By exploiting host cell processes, these enteric pathogens induce their internalization into the host cell by macropinocytosis, at which point other virulence factors may be involved in pathogenesis.

After *C. jejuni* has crossed the mucosal layer and adhered to the underlying intestinal epithelium, the pathogen may invade the intestinal epithelial cells. The process of invasion is responsible for the mucosal damage and inflammatory lesions seen in *C. jejuni* infections and is a major component of *C. jejuni* pathogenesis (162). Invasive strains may account for clinical symptoms such as bloody diarrhea in severe clinical infections. However, not all strains of *C. jejuni* are invasive and even among the invasive sub-types, the frequency of invasion differs substantially. Variation in invasion frequencies may be due to the experimental conditions, including the type and quantity of inoculum and cell cultures used.

Invasion assays for *C. jejuni* have been performed with a number of cell lines, including INT407, HEp-2, and Caco-2 cells, all of intestinal origin (5, 71, 125). Caco-2 cell lines are particularly useful, as they are able to form well differentiated monolayers, with tight junctions and brush border enzymes. In this regard they closely mimic small intestinal epithelial cells, and have been used successfully in studying the invasion and translocation of *Salmonella* (39).

Studies of *C. jejuni* invasion have produced conflicting results. Approximately 5% of a mid-log phase inoculum of *C. jejuni* 81-176 is internalized into semi-confluent monolayers of INT407 or Caco-2 cells in two hours, under optimized conditions for the invasion assay (75). Konkel (71) found that *C. jejuni* invades HEp-2 cells at levels similar to *Salmonella typhimurium*, whereas Russell and Blake (125) found *C. jejuni* was internalized into Caco-2 cells at a higher frequency than *S. typhimurium* (77% to 52% of inoculum, respectively). Overall, the level of *C. jejuni* invasion appears to be comparable to *Yersinia* and *Salmonella typhimurium* (75). *C. jejuni* translocation of polarized Caco-2 cell monolayers has also been demonstrated, but at levels approximately two magnitudes lower than *S. typhimurium* (74).

Co-cultivation of *C. jejuni* with some cell lines can lead to the secretion of proteins, which are proposed to play a role in invasion. The CiaB protein (for *C*ampylobacter *i*nvasion *a*ntigen) is secreted by *C. jejuni* upon co-cultivation with INT407 cells, and although the *ciaB* mutant adheres at similar levels to the wild type, invasion was significantly reduced (73). Up to eight proteins may be secreted in this type of co-cultivation, but their identity and functions have not been determined. Expression of the *cia* genes is regulated by environmental stimuli, such as host intestinal cell components (121). Konkel *et al*. (73) have proposed that CiaB may be a part of a Type III secretion system (T3SS), but Pallen *et al*. (112) challenge this assertion.

A significant amount of evidence suggests *C. jejuni* internalization is dependent on microtubuoles (MT) and microfilaments (MF). Hu and Kopecko (60) have proposed that the entry of *C. jejuni* into host cells is a highly regulated and targeted process, involving vacuole movement along host cell MT. In a model for *C. jejuni* pathogenesis, Kopecko *et al*. (75) propose that the bacterial cell first adheres to the apical surface of intestinal epithelial cells and secrete invasion effector proteins that trigger the extension of MT to form a membrane protrusion. *C. jejuni* is then internalized and remains in the endocytotic vacuole, which may exocytose the bacterium at the baso-lateral surface of the host cell.

At this point, *C. jejuni* may re-invade the cells at the baso-lateral surface, which contains many of the receptors important in invasion (75) and has been demonstrated experimentally (103). M-cells are significant portals of entry for bacterial enteric pathogens (164) and may be the primary point of entry for *C. jejuni* (75), although this has not yet been confirmed.

Overall, there is substantial evidence that invasion is a critical process in *C. jejuni* inflammatory gastroenteritis. This conclusion is supported by data from human clinical infections, human and animal experimental infections, and *in vitro* adherence and invasion assays (61). However, it is important to note that only some *C. jejuni* strains are invasive and that different mechanisms of pathogenesis may be present in different subsets of the *C. jejuni* population (61).

1.9.5 Other putative virulence factors

Iron acquisition, oxidative stress defence, heat shock response and surface polysaccharides have also been identified as potential factors in *C. jejuni* pathogenesis (162), but their role is not yet clearly defined.

1.10 Virulence plasmids in bacterial enteric pathogens

Plasmids are known to play major roles in the pathogenesis of a number of bacterial enteric pathogens, including *Shigella* sp. (129), *Salmonella* sp. (50, 51), and enteropathogenic *Escherichia coli* (66). Falkow (35) has noted that enteric pathogens carry the majority of their essential virulence determinants on extra-chromosomal elements, such as plasmids. Virulence plasmids are especially important in the pathogenicity of enteric pathogens for invasion and adherence.

In *Shigella*, a 220 kb virulence plasmid encodes the proteins necessary for the invasion of the intestinal mucosa. The Ipa proteins (for *I*nvasion *p*lasmid *a*ntigen) are secreted effector proteins that induce cytoskeletal rearrangements in the host intestinal epithelial cells (128). Ipas are secreted by T3SS, which is composed of a number of plasmid-encoded proteins, named Mxi (for *M*embrane e*x*pression of *i*nvasion) and Spa (*S*urface *p*lasmid *a*ntigens). The Mxi and Spa proteins assemble into a needle-like structure in the bacterial cell envelope and act to translocate the Ipa invasins (41). Thus, all of the plasmid encoded components, including the T3SS and the effector proteins it secretes, are required for bacterial invasion of the intestinal epithelium. Similar T3SS are encoded on a plasmid (pYV) in the food-borne pathogen, *Yersinia enterocolitica* (25) and on a pathogenicity island in both *Salmonella* and shiga-toxigenic *E. coli* (40).✓

In addition to the secretion of factors for the invasion of eukaryotic host cells, plasmids can encode virulence factors that act in bacterial adherence to host cells. The 69-kb pEAF plasmid of enteropathogenic *E. coli* (EPEC) encodes the bundle-forming pili (BFP) which are produced in adherence of EPEC and stabilize the attachment of the microcolony to the epithelial surface of the small intestine (155). *E. coli* O157:H7, which is the best known serotype of enterohemmorhagic *E.coli* (EHEC), carries a ~90 kb plasmid, but its role in pathogenesis is unresolved (66). Other types of *E. coli* pathogens, like enteroînvasive *E. coli* (EIEC) and enteroaggregative *E.coli* (EAEC), also carry virulence plasmids (84, 110). However, none of these plasmids have been found in *C. jejuni*.

1.11 *Virulence plasmids in* C. jejuni

Until recently, plasmids were not thought to be involved in the pathogenesis of *Campylobacter* sp. As argued by Bacon *et al*. (5), this long-standing belief was based upon two main lines of evidence: 1) plasmids are found in relatively low levels among pathogenic isolates of *C. jejuni*, and 2) the presence of plasmids does not correlate with pathogenicity, as determined by number of abortions in the pregnant guinea pig model (144).

Thus, compelling evidence for the involvement of plasmids in the virulence of *C. jejuni* was lacking until a plasmid, pVir, was identified in strain 81-176 (5). This ~37.5 kb plasmid contains components of a type IV secretion system (T4SS) (6), which are known to be central to the virulence of a number of major bacterial pathogens, including *Bordetella pertussis*, *Legionella pneumophilia* and *Helicobacter pylori* (20).

T4SSs are macromolecular transfer systems that are ancestrally related to the bacterial conjugation apparatus used for the intercellular transfer of plasmids. Bacterial pathogens use T4SSs to deliver effector proteins into eukaryotic host cells (20). For example, the intracellular pathogen, *L. pneumophilia*, uses a T4SS to secrete proteins that aid in survival within macrophages. In contrast, *B. pertussis* uses a T4SS to secrete pertussis toxin.

The complete DNA sequence of pVir has been determined (6), revealing the presence of seven genes that encode orthologues of T4SS proteins. These pVir genes and their predicted proteins share greatest similarity to the T4SS of *H. pylori*. Mutational analysis of pVir genes has identified nine genes that are involved in the invasion of INT407 cells, an intestinal epithelial cell line (6). *In vitro* insertional mutagenesis of one putative T4SS gene, *cjp5* (a *virB11* homolog) resulted in a six- and eleven-fold reduction in adherence and invasion levels, respectively (5). The *virB11*-type genes encode nucleotide-binding proteins that are proposed to provide the energy for secretion of macromolecules in T4SS (20). Interestingly, mutagenesis of a gene encoding the coupling protein (*cjp4*), which is proposed to assist in docking the substrate for secretion to the T4SS transfer apparatus, did not affect invasion frequencies.

In addition to being important in adherence and invasion *in vitro*, a mutation in *cjp5* reduced the virulence of *C. jejuni* 81-176 in the ferret disease model (5). Overall, *in vitro* evidence from invasion assays with intestinal epithelial cell cultures and *in vivo* data from virulence in ferrets, suggests pVir may be important for the pathogenesis of *C. jejuni*. However, data from the impact of pVir in human clinical infections of *C. jejuni* is lacking.

Fresh clinical isolates of *C. jejuni* have previously been screened for the presence of pVir by probing colony blots with a ^{32}P-labelled *cjp005/virB11* DNA probe. Currently, the only known study of pVir incidence in *C. jejuni* populations has found 10% (*n*=58) of clinical isolates carry pVir (5). Another study has used a DNA microarray for epidemiological studies of *C. jejuni*, and pVir was found at a low rate that the authours did not report (83). By reviewing human feeding studies, Bacon *et al*. (5) noted that *C. jejuni* isolates lacking pVir produced less severe clinical symptoms than infection with 81-176. Other than *C. jejuni* strain 81-176, it is currently unknown if the *C. jejuni* clinical strains that carry pVir are more invasive or more virulent. The role of pVir in the clinical outcome of infection with *C. jejuni* remains unclear.

1.12 Research Objectives

Plasmids play a major role in the dissemination of tetracycline resistance in *C. jejuni* and have been proposed to play a significant role in pathogenesis. This thesis focuses on human clinical isolates of the food-borne enteric pathogen, *C. jejuni*. The research in this thesis is divided into two main topics: 1) antibiotic resistance, and 2) virulence plasmids in *C. jejuni*.

The specific aim of Chapter 2 was to analyze antibiotic resistance in human clinical isolates of *C. jejuni* in Alberta, Canada. Increasing frequencies of tetracycline resistance and the low frequencies of ciprofloxacin and erythromycin resistance were examined, as well as analysis of isolates that were resistant to extremely high levels of tetracycline. The role of resistance plasmids and the *tet*(*O*) gene, in transferring and mediating tetracycline resistance among clinical isolates of *C. jejuni*, has also been investigated.

The importance of the pVir virulence plasmid and antibiotic resistance in the clinical outcomes of *C. jejuni* infection has been evaluated in Chapter 3.

The specific aim of this chapter was to determine the incidence of pVir in clinical isolates of *C. jejuni* and compare pVir incidence to the duration and severity of clinical symptoms. Furthermore, a correlation between pVir incidence and tetracycline resistance in clinical isolates was obtained.

This thesis reports new information in three main areas: 1) the prevalence of antibiotic resistance in human clinical isolates of *C. jejuni* from Alberta, 2) the role of plasmids in conferring high-levels of tetracycline resistance, and 3) the clinical importance of the proposed virulence plasmid, pVir. The results presented herein contribute to the growing body of surveillance data on antibiotic resistance in *C. jejuni*. This research is the first in the field to suggest that the virulence plasmid pVir may be clinically relevant.

Chapter 2

Antibiotic resistance in *C. jejuni*

2 Antibiotic resistance in *C. jejuni*

2.1 Introduction

C. jejuni is a leading cause of bacterial gastroenteritis (99), a disease condition primarily characterized by diarrhea, abdominal pain and fever. *C. jejuni* gastroenteritis is primarily self-limiting and is commonly treated by replacing fluids lost through diarrhea (133). Erythromycin is the drug of choice for treating severe clinical infections with *C. jejuni* (107). Furthermore, ciprofloxacin is commonly prescribed for prophylaxis of enteric infections before travel.

Increased antibiotic resistance is being reported in *C. jejuni*, particularly tetracycline and ciprofloxacin resistance (107). Worldwide, tetracycline-resistance frequencies among human isolates of *C. jejuni* are high, with frequencies of 55 to 56% in North America (43, 105) and up to 95% in Thailand (87). Ciprofloxacin- resistance rates in *C. jejuni* have increased dramatically in the last few decades, reaching up to 88% in Spain (123). Fortunately, the prevalence of erythromycin resistance has remained low, often well below 10% of isolates (108).

In Alberta, Canada, tetracycline-resistance frequencies in human clinical isolates of *C. jejuni* were 6.8% and 8.6% in 1980 and 1981, respectively (145). No erythromycin-resistant isolates were identified in the early 1980's, but resistant isolates have been studied in Alberta by Taylor and colleagues. There is no information on ciprofloxacin resistance frequencies in Alberta among human clinical isolates of *C. jejuni*.

There are several main mechanisms of antibiotic resistance in *C. jejuni*. Resistance to the ciprofloxacin in *C. jejuni* is due to a single base pair mutation in the *gyrA* subunit of the DNA gyrase (topoisomerase II) enzyme (31). Tetracycline resistance in *C. jejuni* is primarily mediated by a plasmid-encoded *tet*(*O*) gene (148), which encodes a ribosomal protection protein (RPP). The *tet*(*O*) gene was originally identified from clinical isolates of *C. jejuni*, but its overall incidence has not been established. Previous studies have determined the *tet*(*O*) gene in *C. jejuni* mediates a tetracycline MIC of 128 μg/mL (145). It is likely that the use of fluoroquinolones and tetracyclines in both human treatment and animal agriculture may lead to the widespread occurrence of resistant strains that threaten effective clinical treatment (32, 98).

Plasmids are a major vehicle for the transmission of antibiotic-resistance determinants among bacterial enteric pathogens (29). Plasmid content in human isolates of *C. jejuni* varies from 13 to 52%, the majority being resistance plasmids. The best studied of these plasmids are the tetracycline-resistance plasmids. In some studies of *C. jejuni* isolates from poultry and retail meats, the primary source for human infections, plasmid content was higher than in humans (up to 91%) (13, 81).

This chapter focuses on antibiotic resistance and documents the substantial increase in the prevalence of tetracycline resistance among clinical isolates of *C. jejuni* over the last two decades in Alberta, Canada. Plasmids isolated from tetracycline-resistant isolates were found to mediate higher levels of tetracycline resistance (512 μg/mL) than was previously identified (32-128 μg/mL). The molecular basis of high-levels of *tet*(*O*)-mediated tetracycline resistance was studied by cloning and DNA sequence analysis. Distinct substitutions were identified in *tet*(*O*) genes from high-level tetracycline-resistant isolates (512 μg/mL) in comparison to *tet*(*O*) genes encoding lower tetracycline-resistance levels, including the *tet*(*O*) gene sequence in GenBank. However, the precise explanation for the molecular basis of this higher level of resistance has not yet been identified.

2.2 Material and Methods

2.2.1 Bacterial strains and plasmids used in this study

A total of 203 *C. jejuni* clinical isolates were obtained from the Alberta Provincial Laboratory for Public Health (APLPH). These included frozen clinical isolates (*n*=193) from 1999 to 2001 and fresh clinical isolates (*n*=10) from 2002. Original isolation and identification of *C. jejuni* isolates was performed by the staff of the APLPH. Strains and plasmids used in this thesis are listed in Table 2.1. Various *C. jejuni* strains with previously characterized tetracycline MIC levels were used as controls. Plasmids pMS119EH, pRY107, and pRY108 were used for cloning experiments.

2.2.2 DNA manipulations

Molecular biology techniques were performed as previously described (127). Restriction enzymes were used as per manufacturer's instructions.

Table 2.1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Selective Marker(s)[a]	Reference or Source
E. coli			
DH5α		Nal	Invitrogen
DY330-N		Nal	(80)
JM109			Stratagene
C. jejuni			
81-176	Contains virulence plasmid pVir	Tc	(5)
UA 543		Nal	Taylor lab strain
UA 543-S		Nal, Sm	Taylor lab strain
UA 56		Tc (MIC=256μg/mL)	Taylor lab strain
UA 143		Tc (MIC=64μg/mL)	Taylor lab strain
UA 183		Tc (MIC=32μg/mL)	Taylor lab strain
***C. jejuni* clinical isolates**			
16-42		Tc (MIC=512μg/mL)	APLPH
23-51		Tc (MIC=512μg/mL)	APLPH
25-01		Tc (MIC=512μg/mL)	APLPH
25-10		Tc (MIC=512μg/mL)	APLPH
23-49		Tc (MIC=128μg/mL)	APLPH
25-44		Tc (MIC=64μg/mL)	APLPH
25-54		Tc (MIC=64μg/mL)	APLPH
Other isolates		Various	APLPH
Plasmids			
pJI3	pACYC177 carrying *tet*(*M*)	Tc	(15)
pMS119EH	Cloning vector, P_{tac}-lacI; pMB1 origin of replication	Amp	(137)
pRY107	*C. jejuni* shuttle vector	Km	(179)
pRY108	*C. jejuni* shuttle vector	Km	(179)
pUOA13	*aph*A-3 kanamycin resistance cassette	Km	(168)

pDOB29	*tet*(O) from 25-01 cloned into pMS119EH	Tc Amp	This study
pDOB32	*tet*(O) from 25-10 cloned into pMS119EH	Tc Amp	This study
pDOB25	*tet*(O) from 16-42 cloned into pMS119EH	Tc Amp	This study
pDOB27	*tet*(O) from 23-51 cloned into pMS119EH	Tc Amp	This study
pDOB31	*tet*(O) from 23-49 cloned into pMS119EH	Tc Amp	This study
pDOB35	*tet*(O) from 25-44 cloned into pMS119EH	Tc Amp	This study
pDOB34	*tet*(O) from 25-54 cloned into pMS119EH	Tc Amp	This study
pDOB42	Kanamycin resistance cassette inserted into *tet*(*O*) of pDOB29	Km	This study
pDOB49	*tet*(O) ORF cloned into pRY107	Tc Km	This study
pDOB45	*tet*(O) ORF with promoter P1 cloned into pRY107	Tc Km	This study
pDOB46	*tet*(O) ORF with promoter P1 cloned into pRY108	Tc Km	This study
pDOB50	*tet*(O) ORF with promoters P1 and P2 cloned into pRY107	Tc Km	This study
pDOB51	*tet*(O) ORF with promoters P1 and P2 cloned into pRY108	Tc Km	This study
pDOB47	*tet*(O) ORF with a ~ 1000 bp upstream region cloned into pRY107	Tc Km	This study
pDOB48	*tet*(O) ORF with a ~ 1000 bp upstream region cloned into pRY108	Tc Km	This study

[a] Antibiotic abbreviations: Tc, tetracycline; Km, kanamycin, Amp, ampicillin; Nal, nalidixic acid

2.2.3 Growth and storage conditions

Clinical isolates retrieved from the APLPH from 1999-2001 were originally frozen in skim milk at -70°C. Frozen isolates were inoculated with a sterile cotton swab onto Brain-Heart Infusion (BHI, Difco-Becton-Dickinson, Sparks, MA) agar and incubated under micro-aerobic conditions for 48 hours at 37°C. Fresh isolates were analyzed directly from the original blood agar isolation plate from the APLPH. Revived cultures, fresh isolates and all other campylobacters were stored frozen in BHI broth (Difco) with 20% glycerol at -80°C. Campylobacters were routinely cultured on BHI agar, supplemented with 0.4% yeast extract (Difco), and incubated at 37°C under micro-aerobic conditions for 48 hours.

2.2.4 Antibiotic susceptibility testing and MICs of tetracycline

Antibiotic discs (Oxoid, Nepean, ON) were used in the disc-diffusion method to determine the susceptibility of *C. jejuni* to five antibiotics: tetracycline (30 μg), kanamycin (30 μg), erythromycin (15 μg), chloramphenicol (30 μg) and nalidixic acid (30 μg). All nalidixic acid-resistant isolates were further tested for resistance to ciprofloxacin (1 μg). Suspensions of *C. jejuni* clinical isolates were made in phosphate-buffered saline (PBS; Sigma, St. Louis, MO) up to a McFarland turbidity standard of 0.5 and were spread out in a lawn onto Mueller-Hinton (MH; Difco) agar. Antibiotic discs were applied asceptically with forceps. After a 48 hour incubation at 37°C in micro-aerobic conditions, zones of inhibition were measured as completed by Gaudreau and Gilbert (44).

MICs of tetracycline-resistant isolates were determined by preparing bacterial suspensions in PBS to a McFarland turbidity standard of 0.5 and diluting 1/10. Suspensions were inoculated onto MH agar containing increasing concentrations of tetracycline (Sigma) (32, 64, 128, 256, and 512 μg/mL) with a Steers replicator. The MIC was defined as the antibiotic concentration which completely inhibited bacterial growth after 48 hours of incubation. For *tet*(*O*) clones in *E.coli*, IPTG (0.1 mM; Rose Scientific, Edmonton, AB) was added to tetracycline plates to induce expression from the pMS119EH vector.

2.2.5 Isolation of bacterial plasmids

Plasmids were isolated by Qiagen Mini or Midi Plasmid Kits (Qiagen, Mississauga, ON) by the manufacturer's instructions, by the alkaline lysis method (127) or by a modified protocol for *Helicobacter pylori* (30). For the latter, a plate of cells were re-suspended in TES buffer (10 mM Tris pH 7.2, 1mM EDTA, 15 mM NaCl), gently mixed with potassium xanthogenate-sodium dodecyl sulfate-phenol (XSP) buffer (1% potassium ethyl xanthogenate, 10mM Tris-HCl, pH 7.4, 20 mM EDTA, pH 8.0, 1% sodium dodecyl sulfate (SDS), 800 mM ammonium acetate) and incubated at 65°C for 30 min. The mixture was vortexed and centrifuged at 14,000 g for 10 min and extracted twice with phenol-chloroform. Plasmid DNA was precipitated by adding the aqueous layer to cold ethanol, which was centrifuged for 15 min (14,000 g), the ethanol was decanted and plasmid DNA was re-suspended in TE buffer. Plasmid DNA concentrations were quantified by gel electrophoresis (BioRad, Mississauga, ON) and spectophotometrically (Ultrospec 3000; Pharmacia Biotech, Piscataway, NJ). Plasmid isolations using the De Ungria *et al.* (30) method were performed primarily by Dr. A. Gibreel.

2.2.6 PCR screen for the *tet*(*O*) and *tet*(*M*) genes

Primers DMT 1 and DMT 2 (Invitrogen, Burlington, ON; Table 2.2) were used to amplify a 559-base pair (bp) product within the *tet*(*O*) gene. PCR templates included plasmid DNA, total DNA and a boiled whole-cell preparation. Plasmid DNA was isolated as described above and total DNA was isolated with a Wizard Genomic DNA isolation Kit (Promega, Madison, WI). Boiled whole cells were prepared by boiling a loopful of cells in 100 μL of TE buffer, which was then centrifuged, and the supernatant used for PCR. PCR conditions were as follows: an initial denaturation of 95°C for 1 minute, then 95°C for 1 min, 50°C for 1 min., and 72°C for 1 min., repeated for 30 cycles. PCR was performed in a BioRad thermocycler. Primers CAT 5 and CAT 6 (Invitrogen; Table 2.2) were used to amplify the *tet*(*M*) gene (2000 bp) from plasmids isolated from high-level tetracycline resistant isolates. PCR products were separated on an agarose gel (Gibco, Burlington, ON), stained with ethidium bromide and visualized on a UV transilluminator. Gel images were captured with Kodak Digital Imaging Software.

Table 2.2. Primers used in this study.

Primer	Sequence[a]	Purpose
DMT 1	5' GGCGTTTTGTTTATGTGCG 3'	Amplification of a 559bp fragment of *tet* (*O*) (5' end)
DMT 2	5' ATGGACAACCCGACAGAAGC 3'	Amplification of a 559bp fragment of *tet* (*O*) (3' end)
CAT 5	5' TATATGAATTCAATGAAAATTATTAATATTGGAG 3'	Amplification of *tet* (*M*)
CAT 6	5' TATATGGATCCACTAAGTTATTTTATTGAAC 3'	Amplification of *tet* (*M*)
DOB 3	5' TATATGAATTCAATGAAAATAATTAACTTAGGCATTC 3'	Cloning of *tet* (*O*) into pMS119EH (5' end)
SEAN 20	5' TATATGGATCCTCTAACTTGTGAACATATGCCG 3'	Cloning of *tet* (*O*) into pMS119EH (3' end)
DMT 27	5' GCAGACTTTCGGCTGCTTTC 3'	Sequencing of *tet* (*O*)
SEAN 5	5' ACTGCTCCGTCTAATACG 3'	Sequencing of tet(*O*)
SEAN 6	5' CAGAACTGGAACAGGAAG 3'	Sequencing of tet(*O*)
SEAN 9	5' ATGCACCGCAGGAATATC 3'	Sequencing of *tet* (*O*)
DMT 29	5' GTGAAGCAAAAGGTTGGGCAGC 3'	Sequencing of *tet* (*O*)
DMT 30	5' GCAGACTTTCGGCTGCTTTC 3'	Sequencing of *tet* (*O*)
DMT 20	5' TATAAGCGCTGGATGAGGAGGCAGATTGCC 3'	Cloning of *aphA* -3 kanamycin resistance cassette into *tet* (*O*)
DMT 21	5' TATAAGCGCTCTAAAACAATTCATCCAG 3'	Cloning of *aphA* -3 kanamycin resistance cassette into *tet* (*O*)
DMT 22	5' TATAGGATCCAATGAAAATAATTAACTTAGGCATTC 3'	Cloning of *tet* (*O*) ORF into pRY107/pRY108 (5' end)
DMT 23	5' TATAGGATCCCTGTCAATTTGATAGTGGGAAC 3'	Cloning of *tet* (*O*) ORF and its P1 promoter into pRY107/pRY108 (5' end)
DMT 24	5' TATAGGATCCGCATAAACAGATGATTAGTGG 3'	Cloning of *tet* (*O*) ORF and its P1/P2 promoters into pRY107/pRY108 (5' end)
DMT 25	5' TATAGGATCCGATATCCACTTGGCTTTATC 3'	Cloning of *tet* (*O*) ORF and a ~1000 bp upstream region into pRY107/pRY108 (5' end)
DMT 26	5' TATAGAATTCTTAAGCTAACTTGTGGAACATATGCC 3'	Cloning of *tet* (*O*) into pRY107/pRY108 (3' end)

[a] Restriction enzyme sites are underlined in the primer sequences.

2.2.7 Conjugative transfer of tetracycline-resistance plasmids

UA 543, a nalidixic acid-resistant strain of *C. jejuni*, was used as the recipient strain for attempts at conjugative transfer of tetracycline-resistance plasmids among campylobacters. Inter-species transfer of plasmids was also attempted, using *E. coli* DY330N as a recipient. Using the method of Taylor *et al.* (146), suspensions of donor and recipient strains were mixed in ratios of 1:4 and 1:6, centrifuged and resuspended in 100 μL of LB broth (Difco). Mating suspensions were inoculated onto a 0.22 μm Millipore filter (Millipore Corporation, Nepean, ON) on BHI agar and incubated for 24 hours. Cultures were resuspended in 1 mL of PBS, diluted, and plated onto MH agar with appropriate antibiotic selection. For all transfers of tetracycline-resistance plasmids from high-level tetracycline-resistant *C. jejuni* clinical isolates, transconjugants were selected on 25 μg/mL tetracycline and 100 μg/mL nalidixic acid. PCR on the *tet*(*O*) gene and tetracycline MICs of the transconjugants was performed as described above. Secondary matings of the transconjugants to a streptomycin-resistant mutant of UA543-S were performed as above, with selection of the secondary transconjugants on 25 μg/mL tetracycline and 50 μg/mL streptomycin.

2.2.8 Cloning of the *tet*(*O*) gene

Primers DOB 3 and SEAN 20 (Invitrogen; see Table 2.2) were used to amplify the entire *tet*(*O*) gene from tetracycline-resistance plasmids isolated from *C. jejuni* clinical isolates with an MIC from each level: 64, 128, and 256-

512 μg/mL tetracycline. A *Pfx*-PCR kit (Invitrogen) was used for all PCR amplifications of tet(*O*) for cloning. *Pfx*-PCR amplification of *tet*(*O*) was used to ensure high PCR fidelity, as the *Pfx* DNA polymerase possesses proof-reading ability. PCR conditions were as follows: denaturation for 30 seconds at 94°C, annealing for 45 s at 55°C and extension for 2 min at 68°C, repeated for 30 cycles. The *tet*(O) gene PCR products were purified using a QiaQuick PCR purification kit (Qiagen), cloned into pMS119EH with *Eco*R1 (Gibco) and *Bam*H1 (Gibco), and transformed into *E. coli* DH5α, which had been made competent by calcium chloride preparation (127). Tetracycline MICs were determined for the transformants as described above. The *tet*(*O*) genes were sequenced by a Big Dye terminator v3.0 Cycle sequencing kit (ABI Prism-A&B Biosystems, Foster City, CA). Sequencing reactions were prepared by Trinh Ngo.

2.2.9 Attempts at expression of *tet*(*O*) genes in *C. jejuni*

The *tet*(*O*) gene from a high-level tetracycline-resistant isolate (25-01) was cloned into the *C. jejuni* shuttle vectors pRY107 and pRY108 (179). Four different clones were made, incorporating increasing portions of the *tet*(*O*) promoter region, including the following: (1) the *tet*(*O*) ORF alone, (2) the ORF and promoter P1, (3) the ORF, P1 and promoter P2, and (4) the ORF and a 1000 bp region upstream of the *tet*(*O*) start codon. A *Pfx*-PCR kit (Invitrogen) was used to amplify the *tet*(*O*) ORF and the varying upstream regions using primers listed in Table 2.2, and cloned into the multiple cloning site of the *lacZ* gene in pRY107 and pRY108, which were transformed into *E. coli* DH5α and colonies were selected on LB with kanamycin (50 μg/mL) (Sigma) and X-gal (40 μg/mL) (Rose Scientific). White colonies were screened for the insertion of the PCR product of the desired size and plasmids were isolated with a Qiagen Midi Kit. Electroporation and transformation was also used to attempt to introduce plasmids carrying the *tet*(*O*) genes into the tetracycline-susceptible *C. jejuni* strain, UA543. Tetracycline MICs were determined for strains containing the *tet*(*O*) clones in *E. coli* DH5α.

2.2.10 Statistical analysis

Tetracycline-resistance frequencies in this study and a previous study from Alberta, Canada (145) were compared by the Fisher Exact test.

2.3 Results

2.3.1 Antibiotic resistance frequencies in *C. jejuni* clinical isolates

Antibiotic susceptibility testing (Table 2.3) determined that 49.8% of *C. jejuni* clinical isolates (101 of 203) were resistant to an antibiotic disc containing 30 μg of tetracycline. The incidence of tetracycline resistance in clinical isolates of *C. jejuni* was found to have increased significantly from 8.6% in 1981 to 49.8% in 1999-2002 (p<0.0001) (145) (Table 2.4).

Erythromycin is the drug of choice in treating *C. jejuni* gastroenteritis, although ciprofloxacin is also a popular choice for antibiotic chemotherapy for other gastric pathogens (107). Low frequencies of erythromycin resistance

Table 2.3. Antibiotic resistance rates in human clinical isolates of *C. jejuni* (*n*=203) from Alberta Canada (1999-2002). Antibiotic disc concentrations are listed below the respective antibiotic.

Antibiotic[a]					
Tc	Ery	Km	Cm	Nal	Cp
30 μg	15 μg	30 μg	30 μg	30 μg	1 μg
49.8%	0.5%	2.5%	0.0%	2.5%	2%

[a] Antibiotic abbreviations: Tc, tetracycline; Ery,erythromycin; Km, kanamycin; Cm, chloramphenicol; Nal, nalidixic acid; Cp, ciprofloxacin.

Table 2.4. Tetracycline resistance rates in clinical isolates of *C. jejuni* from Alberta, Canada.

Year(s)	**%**	**Sample size**	**Tc^{r}** [a]	**Source**
1980	6.8	220	15	(145)
1981	8.6	162	14	(145)
1999-2002	49.8	203	101	This study

[a] Tetracycline resistance

were found, as only one of the 203 clinical isolates (0.5%) was resistant to 15 µg of erythromycin in the disc-diffusion method. No isolates were resistant to chloramphenicol, five were resistant to kanamycin and another five to nalidixic acid (2.5%). Isolates of *C. jejuni* are often resistant to both ciprofloxacin and nalidixic acid, as resistance to both antibiotics is mediated by mutations in the *gyrA* subunit of the DNA gyrase enzyme. The five nalidixic acid-resistant isolates were tested for resistance to ciprofloxacin (1 µg) with the antibiotic disc diffusion method. Ciprofloxacin resistance was found in four of the five nalidixic acid-resistant isolates, for an overall rate of 2%.

The agar dilution method assay subsequently determined that all 101 tetracycline-resistant isolates had MICs of 64 to 512 µg/mL tetracycline, with a significant percentage (37%) of isolates having high-levels of tetracycline resistance (256 to 512 µg/mL MIC) (Table 2.5). A number of control strains of *C. jejuni* with known tetracycline MIC levels were used throughout the agar dilution assays, and their MIC levels were consistent with previously measured results (145).

Multi-drug resistance is becoming an increasing problem in *C. jejuni*, which can complicate effective clinical treatment of campylobacteriosis (58). In this study, several multiple drug-resistant isolates were identified, with nine isolates being resistant to at least two different antibiotics. One isolate (Isolate 24-53) was resistant to three antibiotics (tetracycline, kanamycin, and nalidixic acid). All isolates resistant to kanamycin or nalidixic acid were also resistant to tetracycline. Only a single isolate (Isolate 22-50), was resistant only to

Table 2.5. Minimum inhibitory concentrations (MICs) of tetracycline (Tc) resistant human clinical isolates of *C. jejuni* (*n*=101) from Alberta, Canada (1999-2002).

Tc MIC	Isolates
64 µg/mL	18%
128 µg/mL	46%
256 µg/mL	33%
512 µg/mL	4%

erythromycin, so all multi-drug resistant isolates were always resistant to tetracycline. Therefore tetracycline resistance could be considered a marker for "antibiotic resistance" in *C. jejuni*.

2.3.2 Plasmid content in *C. jejuni*

In this study, plasmid isolations were attempted on 141 of the 203 isolates, including all tetracycline-resistant isolates (*n*=101) and 40 tetracycline-susceptible isolates. Plasmids were isolated from 70 of the 141 isolates (50%), with 67% (68 of 101) and 5% (2 of 40) being isolated from tetracycline-resistant isolates and susceptible isolates, respectively. A ~38-40 kb plasmid was present in all four high-level resistant isolates (512 μg/mL). Plasmid size varied from approximately three to 100 kb, with some isolates containing up to four plasmid bands (A. Gibreel, personal communication).

Several methods of plasmid isolation were used to confirm the presence and/or absence of plasmids. This included commercial plasmid preparation kits (Qiagen), a traditional alkaline lysis method (127), and a modified plasmid isolation method described for *H. pylori* (30). Difficulties were encountered with some strains and the use of multiple plasmid isolation methods ensured that the absence of a plasmid was confirmed. Qiagen Mini-plasmid kits were found to be rapid and the most successful method for isolating plasmid DNA from *C. jejuni* clinical isolates.

2.3.3 Location and incidence of the *tet*(*O*) and *tet*(*M*) determinants

A PCR screen on plasmid isolations or boiled whole cells identified the *tet*(*O*) gene in 94% (*n*=95) of the tetracycline-resistant *C. jejuni* isolates (*n*=101), including all high-level resistant isolates (512 μg/mL MIC) (*n*=4) (Figure 2.1). The *tet*(*O*) gene was primarily located on plasmids, but positive results in total DNA preparations were found in isolates lacking plasmids, suggesting the *tet*(*O*) gene can be located on the *C. jejuni* chromosome.

In the six tetracycline-resistant isolates that were not positive for the *tet*(*O*) gene in the PCR screen, DNA-DNA hybridizations on total DNA preparations produced a positive signal with a ^{32}P-labelled *tet*(*O*) probe. None of the high-level tetracycline-resistant isolates (512 μg/mL MIC) contained *tet*(*M*), based upon a PCR screen (Figure 2.2). DNA-DNA hybridizations for the *tet*(*O*) gene were performed by Dr. A. Gibreel. Overall, the *tet*(*O*) gene was identified in all tetracycline-resistant *C. jejuni* isolates either by PCR or DNA hybridizations, mainly on plasmids but occasionally on the chromosome.

2.3.4 Conjugative transfer of plasmids harbouring the *tet*(*O*) gene

Previous research has revealed that tetracycline resistance in *C. jejuni* is mediated by transmissible plasmids (146). To study the basis for high-level tetracycline resistance in four of the *C. jejuni* clinical isolates, mating experiments were performed to transfer plasmids carrying *tet*(*O*) from the high-

Figure 2.1. PCR screen for the *tet*(*O*) gene in *C. jejuni* clinical isolates. PCR amplification of a segment of the *tet*(*O*) gene (559 bp) was achieved from both purified plasmid DNA and boiled whole cell preparation templates. Lane 1: 1 kb DNA ladder; Lane 2: (C, negative control reaction, pMS119EH plasmid DNA); Lane 3: (T, positive control, pTet from UA 1376); Lanes 4-15, PCR results on isolated plasmid DNA from *C. jejuni* clinical isolates.

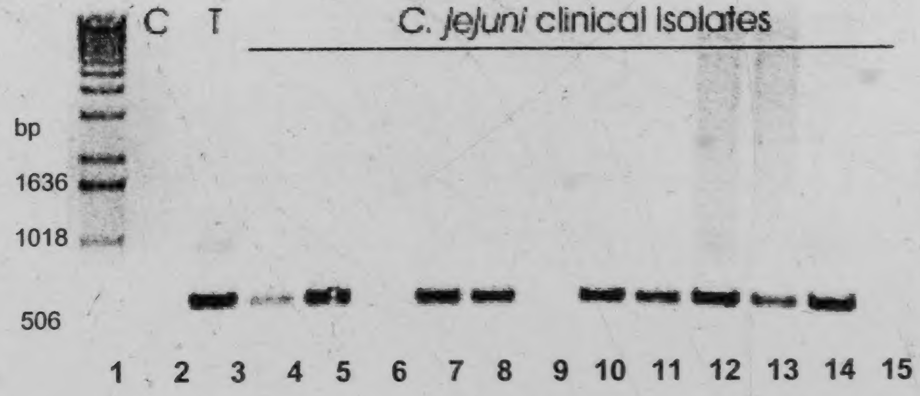
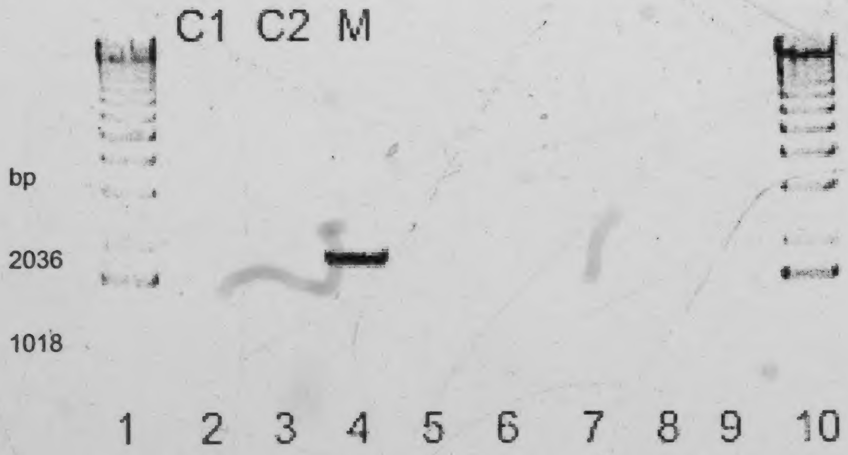

C
T
C. jejuni clinical isolates
bp
1636
1018
506
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 2.2. A PCR screen for the *tet*(*M*) gene in clinical isolates of *C. jejuni* resistant to a high level of tetracycline (512 µg/mL). Lane 1 and 10, 1kb DNA ladder; Lane 2 (C1, negative control, pMS119EH plasmid DNA); Lane 3 (C2, negative control, no DNA); Lane 4 (M, *tet*(*M*) positive control, pJI3); Lane 5, plasmid DNA from 25-01; Lane 6, plasmid DNA from 23-51; Lane 7, plasmid DNA from 25-10; Lane 8, plasmid DNA from 16-42, Lane 9, empty well.


C1 C2 M
bp
2036
1018
1 2 3 4 5 6 7 8 9 10

level tetracycline-resistant *C. jejuni* isolates (512 μg/mL MIC) to tetracycline-susceptible, nalidixic acid-resistant *C. jejuni* recipients (UA543). If the resulting transconjugant had a tetracycline MIC of 512 μg/mL, then the plasmid likely contains the genes responsible for mediating high-level tetracycline resistance.

Conjugative transfer of tetracycline-resistance plasmids between strains of *C. jejuni* has been demonstrated previously (145, 146) and occurs at frequencies of approximately 4 x 10^{-5} transconjugants per recipient in a 24h mating on a solid surface at 37^0C (145). This study confirmed these initial findings, with transfer frequencies of 10^{-4} and 10^{-5} transconjugants per recipient under similar conditions (Table 2.6). Attempts to transfer tetracycline-resistance plasmids from *C. jejuni* isolates to *E. coli* were unsuccessful. Earlier attempts at transferring similar plasmids from *C. jejuni* to *E. coli* were also unsuccessful and suggested the host range was restricted to *Campylobacter* sp. (146).

The *tet*(O) gene PCR screen and isolation of plasmids from tetracycline-resistant transconjugants confirmed transfer of tetracycline-resistance plasmids to recipients. Transconjugants exhibited MICs at levels equal to that of the donor strain (512 μg/mL). These results suggest the plasmids alone encode the resistance determinants mediating high-levels of tetracycline resistance.

2.3.5 Cloning of *tet*(*O*) from high-level tetracycline resistant isolates

To further characterize the molecular basis for high-level tetracycline resistance, *tet*(*O*) genes from various tetracycline MIC levels (64, 128 and 256-512 μg/mL) were cloned from plasmids isolated from *C. jejuni* clinical isolates.

Table 2.6. Conjugative transfer of tetracycline (Tc) resistance plasmids from *C. jejuni* clinical isolates to UA543, a tetracycline-susceptible *C. jejuni* recipient strain.

C. jejuni donor isolate	Donor Tc MIC (μg/mL)	Transfer frequency per recipient	Transconjugant Tc MIC (μg/mL)
16-42	512	3.78×10^{-4}	512
23-51	512	3.75×10^{-5}	512
25-01	512	1.34×10^{-4}	512
25-10	512	7.60×10^{-5}	512

Primers DOB 3 and SEAN 20 were successful in the amplification of *tet*(*O*) and for cloning into pMS119EH (Figure 2.3). The resulting ligations were transformed into *E. coli* DH5α with high-efficiency through the calcium-chloride transformation method (127).

Cloning of *tet*(*O*) has previously been accomplished (93) and the tetracycline MICs in both the original *C. jejuni* isolate and the *E. coli* clones were comparable (143). The *C. jejuni* isolate from which the original *tet*(*O*) gene was cloned (Strain UA 466) had a tetracycline MIC of 64 μg/mL. To determine if the *tet*(*O*) genes from high-level tetracycline-resistant isolates (512 μg/mL) were able to mediate higher levels of resistance in *E. coli*, tetracycline MICs were determined for the *tet*(*O*) clones in DH5α. Tetracycline MIC levels for *E. coli* DH5α transformed with *tet*(*O*) expression vectors (pMS119EH:*tet*(*O*)) are presented in Table 2.7. Although MIC levels differ in the host from which the *tet*(*O*) genes were cloned, they mediated identical levels of tetracycline resistance in *E. coli* DH5α (32 μg/mL). Therefore the difference in resistance levels is specific to a *C. jejuni* genetic background. Furthermore, other research projects in the Taylor laboratory have found that a *tet*(*O*) gene cloned into pMS119EH and transformed into a different *E. coli* strain, JM109, mediated a tetracycline MIC of 128 μg/mL (L. Nonaka, personal communication).

The same expression vectors were used to transform *E. coli* DH5α and *E. coli* JM109. A host-dependent response was found, as the same expression

Figure 2.3. Cloning of the *tet*(*O*) gene into pMS119EH. SC=super coiled ladder, Lane 1, pMS119EH, Lanes 2-6, *tet*(*O*) cloned into pMS119EH (see Materials and Methods).

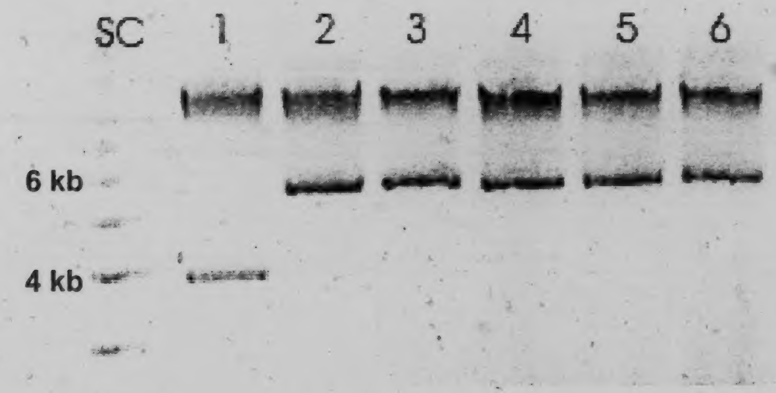

SC
1
2
3
4
5
6
6 kb
4 kb

Table 2.7. Tetracycline (Tc) MICs of *C. jejuni* and of the *tet*(*O*) genes cloned into pMS119EH in *E. coli*.

***C. jejuni* clinical isolate**	**Tc MIC in *C. jejuni***	**Tc MIC in *E. coli* DH5α**	**Tc MIC in *E. coli* JM109**	**Reference**
25-01	512	32	128	This study
23-49	128	32	ND[a]	This study
25-44	64	32	ND[a]	This study
25-54	64	32	ND[a]	This study
UA 466	64	ND[a]	128	(145)

[a] ND=Not determined.

vectors that mediated a tetracycline MIC of 32 μg/mL in DH5α was found to mediate an MIC of 128 μg/mL in JM109.

2.3.6 Sequence and alignments of the *tet*(*O*) gene

DNA sequencing of *tet*(*O*) genes (1.92 kb) from *C. jejuni* clinical isolates with different tetracycline MIC levels was performed to determine if particular mutations might be responsible for higher levels of tetracycline resistance. The sequencing strategy, which shows sequencing primer overlapping, is presented in Figure 2.4. Tetracycline-resistant *C. jejuni* from the 64 and 128 μg/mL tetracycline MIC levels were found to have *tet*(*O*) genes with identical DNA sequences (Figure 2.5). However, a *tet*(*O*) gene from clinical isolate 25-01, which is resistant to 512 μg/mL of tetracycline, had seven nucleotide changes that differed from the *tet*(O) genes encoding lower MIC levels (Table 2.8). An eighth substitution was also found at nucleotide position 1772, but is present in all *tet*(*O*) genes sequenced, suggesting the GenBank *tet*(*O*) sequence is incorrect (S. Connell, personal communication). All *tet*(*O*) genes sequenced had different terminal 3' sequences compared to the GenBank *tet*(*O*) sequence, but were identical to each other. Analysis of this data and of other sequence data from the Taylor lab (L. Nonaka, personal communication; S. Connell, personal communication) has determined that the GenBank *tet*(*O*) sequence is incorrect at the 3' end. These changes have been updated in the GenBank *tet*(*O*) sequence.

Figure 2.4. Strategy for sequencing of the *tet*(*O*) gene. Schematic representation of primer binding to *tet*(*O*) and extension of products, based upon output of SeqMan (DNA-Star/LaserGene).

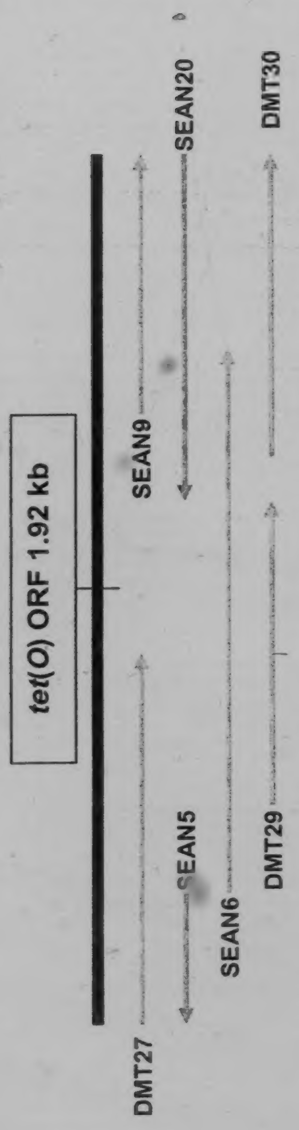
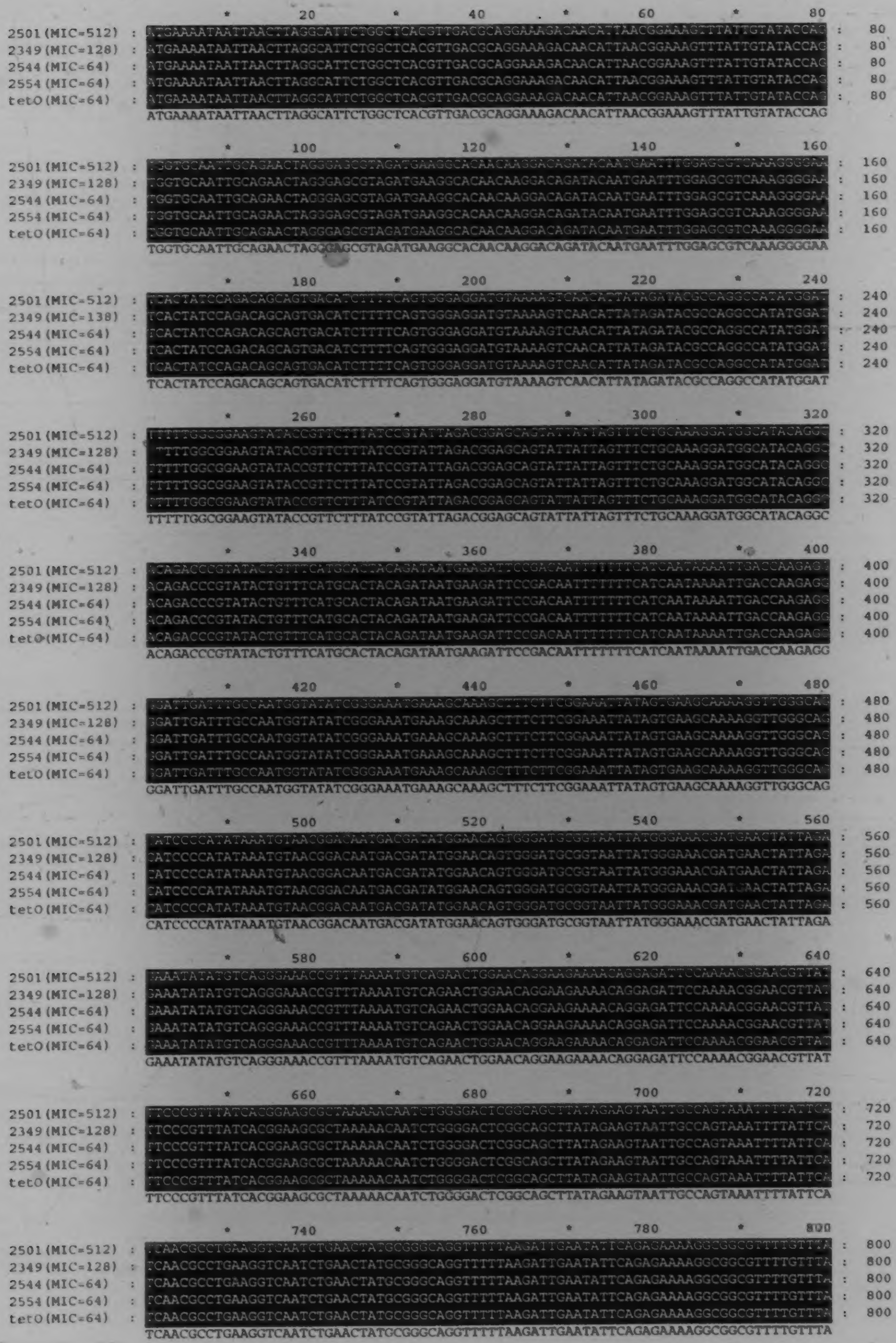

tet(*O*) ORF 1.92 kb
SEAN9
SEAN20
DMT30
DMT27
SEAN5
SEAN6
DMT29

Figure 2.5. Nucleotide sequence alignment of *tet*(*O*) genes(1.92 kb) cloned from *Campylobacter jejuni* clinical isolates with different MICs of tetracycline. Isolate numbers are listed with their respective MIC level (512, 128, or 64 µg/mL). Black shading represents identical sequences and grey shading represents divergent sequences. Sequences were aligned with Lasergene DNA Star software.



```
                           *        20         *        40       .  *        60         *        80
2501(MIC=512)  : ATGAAAATAATTAACTTAGGCATTCTGGCTCACGTTGACGCAGGAAAGACAACATTAACGGAAAGTTTATTGTATACCAG :  80
2349(MIC=128)  : ATGAAAATAATTAACTTAGGCATTCTGGCTCACGTTGACGCAGGAAAGACAACATTAACGGAAAGTTTATTGTATACCAG :  80
2544(MIC=64)   : ATGAAAATAATTAACTTAGGCATTCTGGCTCACGTTGACGCAGGAAAGACAACATTAACGGAAAGTTTATTGTATACCAG :  80
2554(MIC=64)   : ATGAAAATAATTAACTTAGGCATTCTGGCTCACGTTGACGCAGGAAAGACAACATTAACGGAAAGTTTATTGTATACCAG :  80
tetO(MIC=64)   : ATGAAAATAATTAACTTAGGCATTCTGGCTCACGTTGACGCAGGAAAGACAACATTAACGGAAAGTTTATTGTATACCAG :  80
                 ATGAAAATAATTAACTTAGGCATTCTGGCTCACGTTGACGCAGGAAAGACAACATTAACGGAAAGTTTATTGTATACCAG

                           *       100         *       120         *       140         *       160
2501(MIC=512)  : TGGTGCAATTGCAGAACTAGGGAGCGTAGATGAAGGCACAACAAGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAA : 160
2349(MIC=128)  : TGGTGCAATTGCAGAACTAGGGAGCGTAGATGAAGGCACAACAAGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAA : 160
2544(MIC=64)   : TGGTGCAATTGCAGAACTAGGGAGCGTAGATGAAGGCACAACAAGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAA : 160
2554(MIC=64)   : TGGTGCAATTGCAGAACTAGGGAGCGTAGATGAAGGCACAACAAGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAA : 160
tetO(MIC=64)   : TGGTGCAATTGCAGAACTAGGGAGCGTAGATGAAGGCACAACAAGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAA : 160
                 TGGTGCAATTGCAGAACTAGGGAGCGTAGATGAAGGCACAACAAGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAA

                           *       180         *       200         *       220         *       240
2501(MIC=512)  : TCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGTAAAAGTCAACATTATAGATACGCCAGGCCATATGGAT : 240
2349(MIC=138)  : TCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGTAAAAGTCAACATTATAGATACGCCAGGCCATATGGAT : 240
2544(MIC=64)   : TCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGTAAAAGTCAACATTATAGATACGCCAGGCCATATGGAT : 240
2554(MIC=64)   : TCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGTAAAAGTCAACATTATAGATACGCCAGGCCATATGGAT : 240
tetO(MIC=64)   : TCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGTAAAAGTCAACATTATAGATACGCCAGGCCATATGGAT : 240
                 TCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGTAAAAGTCAACATTATAGATACGCCAGGCCATATGGAT

                           *       260         *       280         *       300         *       320
2501(MIC=512)  : TTTTTGGCGGAAGTATACCGTTCTTTATCCGTATTAGACGGAGCAGTATTATTAGTTTCTGCAAAGGATGGCATACAGGC : 320
2349(MIC=128)  :  TTTTGGCGGAAGTATACCGTTCTTTATCCGTATTAGACGGAGCAGTATTATTAGTTTCTGCAAAGGATGGCATACAGGC : 320
2544(MIC=64)   : TTTTTGGCGGAAGTATACCGTTCTTTATCCGTATTAGACGGAGCAGTATTATTAGTTTCTGCAAAGGATGGCATACAGGC : 320
2554(MIC=64)   : TTTTTGGCGGAAGTATACCGTTCTTTATCCGTATTAGACGGAGCAGTATTATTAGTTTCTGCAAAGGATGGCATACAGGC : 320
tetO(MIC=64)   : TTTTTGGCGGAAGTATACCGTTCTTTATCCGTATTAGACGGAGCAGTATTATTAGTTTCTGCAAAGGATGGCATACAGGC : 320
                 TTTTTGGCGGAAGTATACCGTTCTTTATCCGTATTAGACGGAGCAGTATTATTAGTTTCTGCAAAGGATGGCATACAGGC

                           *       340         *       360         *       380         *       400
2501(MIC=512)  : ACAGACCCGTATACTGTTTCATGCACTACAGATAATGAAGATTCCGACAATTTTTTTCATCAATAAAATTGACCAAGAGG : 400
2349(MIC=128)  : ACAGACCCGTATACTGTTTCATGCACTACAGATAATGAAGATTCCGACAATTTTTTTCATCAATAAAATTGACCAAGAGG : 400
2544(MIC=64)   : ACAGACCCGTATACTGTTTCATGCACTACAGATAATGAAGATTCCGACAATTTTTTTCATCAATAAAATTGACCAAGAGG : 400
2554(MIC=64)   : ACAGACCCGTATACTGTTTCATGCACTACAGATAATGAAGATTCCGACAATTTTTTTCATCAATAAAATTGACCAAGAGG : 400
tetO(MIC=64)   : ACAGACCCGTATACTGTTTCATGCACTACAGATAATGAAGATTCCGACAATTTTTTTCATCAATAAAATTGACCAAGAGG : 400
                 ACAGACCCGTATACTGTTTCATGCACTACAGATAATGAAGATTCCGACAATTTTTTTCATCAATAAAATTGACCAAGAGG

                           *       420         *       440         *       460         *       480
2501(MIC=512)  : GGATTGATTTGCCAATGGTATATCGGGAAATGAAAGCAAAGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAG : 480
2349(MIC=128)  : GGATTGATTTGCCAATGGTATATCGGGAAATGAAAGCAAAGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAG : 480
2544(MIC=64)   : GGATTGATTTGCCAATGGTATATCGGGAAATGAAAGCAAAGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAG : 480
2554(MIC=64)   : GGATTGATTTGCCAATGGTATATCGGGAAATGAAAGCAAAGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAG : 480
tetO(MIC=64)   : GGATTGATTTGCCAATGGTATATCGGGAAATGAAAGCAAAGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAG : 480
                 GGATTGATTTGCCAATGGTATATCGGGAAATGAAAGCAAAGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAG

                           *       500         *       520         *       540         *       560
2501(MIC=512)  : CATCCCCATATAAATGTAACGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAGA : 560
2349(MIC=128)  : CATCCCCATATAAATGTAACGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAGA : 560
2544(MIC=64)   : CATCCCCATATAAATGTAACGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAGA : 560
2554(MIC=64)   : CATCCCCATATAAATGTAACGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAGA : 560
tetO(MIC=64)   : CATCCCCATATAAATGTAACGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAGA : 560
                 CATCCCCATATAAATGTAACGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAGA

                           *       580         *       600         *       620         *       640
2501(MIC=512)  : GAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGAGATTCCAAAACGGAACGTTAT : 640
2349(MIC=128)  : GAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGAGATTCCAAAACGGAACGTTAT : 640
2544(MIC=64)   : GAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGAGATTCCAAAACGGAACGTTAT : 640
2554(MIC=64)   : GAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGAGATTCCAAAACGGAACGTTAT : 640
tetO(MIC=64)   : GAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGAGATTCCAAAACGGAACGTTAT : 640
                 GAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGAGATTCCAAAACGGAACGTTAT

                           *       660         *       680         *       700         *       720
2501(MIC=512)  : TTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGACTCGGCAGCTTATAGAAGTAATTGCCAGTAAATTTTATTCA : 720
2349(MIC=128)  : TTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGACTCGGCAGCTTATAGAAGTAATTGCCAGTAAATTTTATTCA : 720
2544(MIC=64)   : TTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGACTCGGCAGCTTATAGAAGTAATTGCCAGTAAATTTTATTCA : 720
2554(MIC=64)   : TTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGACTCGGCAGCTTATAGAAGTAATTGCCAGTAAATTTTATTCA : 720
tetO(MIC=64)   : TTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGACTCGGCAGCTTATAGAAGTAATTGCCAGTAAATTTTATTCA : 720
                 TTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGACTCGGCAGCTTATAGAAGTAATTGCCAGTAAATTTTATTCA

                           *       740         *       760         *       780         *       800
2501(MIC=512)  : TCAACGCCTGAAGGTCAATCTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTTA : 800
2349(MIC=128)  : TCAACGCCTGAAGGTCAATCTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTTA : 800
2544(MIC=64)   : TCAACGCCTGAAGGTCAATCTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTTA : 800
2554(MIC=64)   : TCAACGCCTGAAGGTCAATCTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTTA : 800
tetO(MIC=64)   : TCAACGCCTGAAGGTCAATCTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTTA : 800
                 TCAACGCCTGAAGGTCAATCTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTTA
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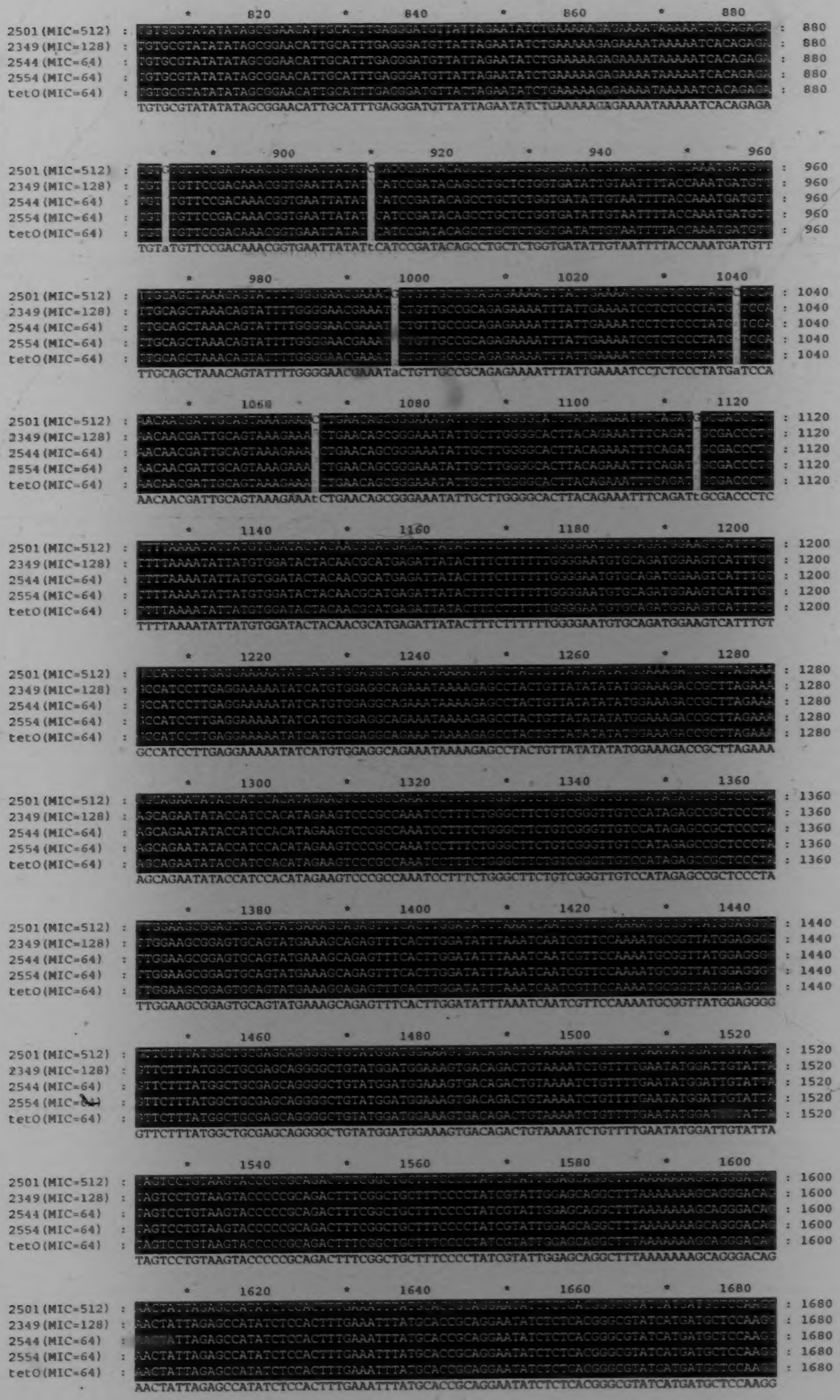

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                          *      820        *      840        *      860        *      880
2501(MIC=512) : TGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAGAGAAAATAAAAATCACAGAGA :  880
2349(MIC=128) : TGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAGAGAAAATAAAAATCACAGAGA :  880
2544(MIC=64)  : TGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAGAGAAAATAAAAATCACAGAGA :  880
2554(MIC=64)  : TGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAGAGAAAATAAAAATCACAGAGA :  880
tetO(MIC=64)  : TGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAGAGAAAATAAAAATCACAGAGA :  880
                TGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAGAGAAAATAAAAATCACAGAGA

                          *      900        *      920        *      940        *      960
2501(MIC=512) : TGTATGTTCCGACAAACGGTGAATTATATTCATCCGATACAGCCTGCTCTGGTGATATTGTAATTTTACCAAATGATGTT :  960
2349(MIC=128) : TGT TGTTCCGACAAACGGTGAATTATAT CATCCGATACAGCCTGCTCTGGTGATATTGTAATTTTACCAAATGATGTT :  960
2544(MIC=64)  : TGT TGTTCCGACAAACGGTGAATTATAT CATCCGATACAGCCTGCTCTGGTGATATTGTAATTTTACCAAATGATGTT :  960
2554(MIC=64)  : TGT TGTTCCGACAAACGGTGAATTATAT CATCCGATACAGCCTGCTCTGGTGATATTGTAATTTTACCAAATGATGTT :  960
tetO(MIC=64)  : TGT TGTTCCGACAAACGGTGAATTATAT CATCCGATACAGCCTGCTCTGGTGATATTGTAATTTTACCAAATGATGTT :  960
                TGTaTGTTCCGACAAACGGTGAATTATATtCATCCGATACAGCCTGCTCTGGTGATATTGTAATTTTACCAAATGATGTT

                          *      980        *     1000        *     1020        *     1040
2501(MIC=512) : TTGCAGCTAAACAGTATTTTGGGGAACGAAATACTGTTGCCGCAGAGAAAATTTATTGAAAATCCTCTCCCTATGATCCA : 1040
2349(MIC=128) : TTGCAGCTAAACAGTATTTTGGGGAACGAAAT CTGTTGCCGCAGAGAAAATTTATTGAAAATCCTCTCCCTATG TCCA : 1040
2544(MIC=64)  : TTGCAGCTAAACAGTATTTTGGGGAACGAAAT CTGTTGCCGCAGAGAAAATTTATTGAAAATCCTCTCCCTATG TCCA : 1040
2554(MIC=64)  : TTGCAGCTAAACAGTATTTTGGGGAACGAAAT CTGTTGCCGCAGAGAAAATTTATTGAAAATCCTCTCCCTATG TCCA : 1040
tetO(MIC=64)  : TTGCAGCTAAACAGTATTTTGGGGAACGAAAT CTGTTGCCGCAGAGAAAATTTATTGAAAATCCTCTCCCTATG TCCA : 1040
                TTGCAGCTAAACAGTATTTTGGGGAACGAAATaCTGTTGCCGCAGAGAAAATTTATTGAAAATCCTCTCCCTATGaTCCA

                          *     1060        *     1080        *     1100        *     1120
2501(MIC=512) : AACAACGATTGCAGTAAAGAAATCTGAACAGCGGGAAATATTGCTTGGGGCACTTACAGAAATTTCAGATTGCGACCCTC : 1120
2349(MIC=128) : AACAACGATTGCAGTAAAGAAA CTGAACAGCGGGAAATATTGCTTGGGGCACTTACAGAAATTTCAGAT GCGACCCTC : 1120
2544(MIC=64)  : AACAACGATTGCAGTAAAGAAA CTGAACAGCGGGAAATATTGCTTGGGGCACTTACAGAAATTTCAGAT GCGACCCTC : 1120
2554(MIC=64)  : AACAACGATTGCAGTAAAGAAA CTGAACAGCGGGAAATATTGCTTGGGGCACTTACAGAAATTTCAGAT GCGACCCTC : 1120
tetO(MIC=64)  : AACAACGATTGCAGTAAAGAAA CTGAACAGCGGGAAATATTGCTTGGGGCACTTACAGAAATTTCAGAT GCGACCCTC : 1120
                AACAACGATTGCAGTAAAGAAAtCTGAACAGCGGGAAATATTGCTTGGGGCACTTACAGAAATTTCAGATtGCGACCCTC

                          *     1140        *     1160        *     1180        *     1200
2501(MIC=512) : TTTTAAAATATTATGTGGATACTACAACGCATGAGATTATACTTTCTTTTTTGGGGAATGTGCAGATGGAAGTCATTTGT : 1200
2349(MIC=128) : TTTTAAAATATTATGTGGATACTACAACGCATGAGATTATACTTTCTTTTTTGGGGAATGTGCAGATGGAAGTCATTTGT : 1200
2544(MIC=64)  : TTTTAAAATATTATGTGGATACTACAACGCATGAGATTATACTTTCTTTTTTGGGGAATGTGCAGATGGAAGTCATTTGT : 1200
2554(MIC=64)  : TTTTAAAATATTATGTGGATACTACAACGCATGAGATTATACTTTCTTTTTTGGGGAATGTGCAGATGGAAGTCATTTGT : 1200
tetO(MIC=64)  : TTTTAAAATATTATGTGGATACTACAACGCATGAGATTATACTTTCTTTTTTGGGGAATGTGCAGATGGAAGTCATTTGT : 1200
                TTTTAAAATATTATGTGGATACTACAACGCATGAGATTATACTTTCTTTTTTGGGGAATGTGCAGATGGAAGTCATTTGT

                          *     1220        *     1240        *     1260        *     1280
2501(MIC=512) : GCCATCCTTGAGGAAAAAATATCATGTGGAGGCAGAAATAAAAGAGCCTACTGTTATATATATGGAAAGACCGCTTAGAAA : 1280
2349(MIC=128) : GCCATCCTTGAGGAAAAAATATCATGTGGAGGCAGAAATAAAAGAGCCTACTGTTATATATATGGAAAGACCGCTTAGAAA : 1280
2544(MIC=64)  : GCCATCCTTGAGGAAAAAATATCATGTGGAGGCAGAAATAAAAGAGCCTACTGTTATATATATGGAAAGACCGCTTAGAAA : 1280
2554(MIC=64)  : GCCATCCTTGAGGAAAAAATATCATGTGGAGGCAGAAATAAAAGAGCCTACTGTTATATATATGGAAAGACCGCTTAGAAA : 1280
tetO(MIC=64)  : GCCATCCTTGAGGAAAAAATATCATGTGGAGGCAGAAATAAAAGAGCCTACTGTTATATATATGGAAAGACCGCTTAGAAA : 1280
                GCCATCCTTGAGGAAAAAATATCATGTGGAGGCAGAAATAAAAGAGCCTACTGTTATATATATGGAAAGACCGCTTAGAAA

                          *     1300        *     1320        *     1340        *     1360
2501(MIC=512) : AGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCATAGAGCCGCTCCCTA : 1360
2349(MIC=128) : AGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCATAGAGCCGCTCCCTA : 1360
2544(MIC=64)  : AGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCATAGAGCCGCTCCCTA : 1360
2554(MIC=64)  : AGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCATAGAGCCGCTCCCTA : 1360
tetO(MIC=64)  : AGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCATAGAGCCGCTCCCTA : 1360
                AGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCATAGAGCCGCTCCCTA

                          *     1380        *     1400        *     1420        *     1440
2501(MIC=512) : TTGGAAGCGGAGTGCAGTATGAAAGCAGAGTTTCACTTGGATATTTAAATCAATCGTTCCAAAATGCGGTTATGGAGGGG : 1440
2349(MIC=128) : TTGGAAGCGGAGTGCAGTATGAAAGCAGAGTTTCACTTGGATATTTAAATCAATCGTTCCAAAATGCGGTTATGGAGGGG : 1440
2544(MIC=64)  : TTGGAAGCGGAGTGCAGTATGAAAGCAGAGTTTCACTTGGATATTTAAATCAATCGTTCCAAAATGCGGTTATGGAGGGG : 1440
2554(MIC=64)  : TTGGAAGCGGAGTGCAGTATGAAAGCAGAGTTTCACTTGGATATTTAAATCAATCGTTCCAAAATGCGGTTATGGAGGGG : 1440
tetO(MIC=64)  : TTGGAAGCGGAGTGCAGTATGAAAGCAGAGTTTCACTTGGATATTTAAATCAATCGTTCCAAAATGCGGTTATGGAGGGG : 1440
                TTGGAAGCGGAGTGCAGTATGAAAGCAGAGTTTCACTTGGATATTTAAATCAATCGTTCCAAAATGCGGTTATGGAGGGG

                          *     1460        *     1480        *     1500        *     1520
2501(MIC=512) : GTTCTTTATGGCTGCGAGCAGGGGCTGTATGGATGGAAAGTGACAGACTGTAAAATCTGTTTTGAATATGGATTGTATTA : 1520
2349(MIC=128) : GTTCTTTATGGCTGCGAGCAGGGGCTGTATGGATGGAAAGTGACAGACTGTAAAATCTGTTTTGAATATGGATTGTATTA : 1520
2544(MIC=64)  : GTTCTTTATGGCTGCGAGCAGGGGCTGTATGGATGGAAAGTGACAGACTGTAAAATCTGTTTTGAATATGGATTGTATTA : 1520
2554(MIC=64)  : GTTCTTTATGGCTGCGAGCAGGGGCTGTATGGATGGAAAGTGACAGACTGTAAAATCTGTTTTGAATATGGATTGTATTA : 1520
tetO(MIC=64)  : GTTCTTTATGGCTGCGAGCAGGGGCTGTATGGATGGAAAGTGACAGACTGTAAAATCTGTTTTGAATATGGATTGTATTA : 1520
                GTTCTTTATGGCTGCGAGCAGGGGCTGTATGGATGGAAAGTGACAGACTGTAAAATCTGTTTTGAATATGGATTGTATTA

                          *     1540        *     1560        *     1580        *     1600
2501(MIC=512) : TAGTCCTGTAAGTACCCCCGCAGACTTTCGGCTGCTTTCCCCTATCGTATTGGAGCAGGCTTTAAAAAAAGCAGGGACAG : 1600
2349(MIC=128) : TAGTCCTGTAAGTACCCCCGCAGACTTTCGGCTGCTTTCCCCTATCGTATTGGAGCAGGCTTTAAAAAAAGCAGGGACAG : 1600
2544(MIC=64)  : TAGTCCTGTAAGTACCCCCGCAGACTTTCGGCTGCTTTCCCCTATCGTATTGGAGCAGGCTTTAAAAAAAGCAGGGACAG : 1600
2554(MIC=64)  : TAGTCCTGTAAGTACCCCCGCAGACTTTCGGCTGCTTTCCCCTATCGTATTGGAGCAGGCTTTAAAAAAAGCAGGGACAG : 1600
tetO(MIC=64)  : TAGTCCTGTAAGTACCCCCGCAGACTTTCGGCTGCTTTCCCCTATCGTATTGGAGCAGGCTTTAAAAAAAGCAGGGACAG : 1600
                TAGTCCTGTAAGTACCCCCGCAGACTTTCGGCTGCTTTCCCCTATCGTATTGGAGCAGGCTTTAAAAAAAGCAGGGACAG

                          *     1620        *     1640        *     1660        *     1680
2501(MIC=512) : AACTATTAGAGCCATATCTCCACTTTGAAATTTATGCACCGCAGGAATATCTCTCACGGGCGTATCATGATGCTCCAAGG : 1680
2349(MIC=128) : AACTATTAGAGCCATATCTCCACTTTGAAATTTATGCACCGCAGGAATATCTCTCACGGGCGTATCATGATGCTCCAAGG : 1680
2544(MIC=64)  : AACTATTAGAGCCATATCTCCACTTTGAAATTTATGCACCGCAGGAATATCTCTCACGGGCGTATCATGATGCTCCAAGG : 1680
2554(MIC=64)  : AACTATTAGAGCCATATCTCCACTTTGAAATTTATGCACCGCAGGAATATCTCTCACGGGCGTATCATGATGCTCCAAGG : 1680
tetO(MIC=64)  : AACTATTAGAGCCATATCTCCACTTTGAAATTTATGCACCGCAGGAATATCTCTCACGGGCGTATCATGATGCTCCAAGG : 1680
                AACTATTAGAGCCATATCTCCACTTTGAAATTTATGCACCGCAGGAATATCTCTCACGGGCGTATCATGATGCTCCAAGG
```
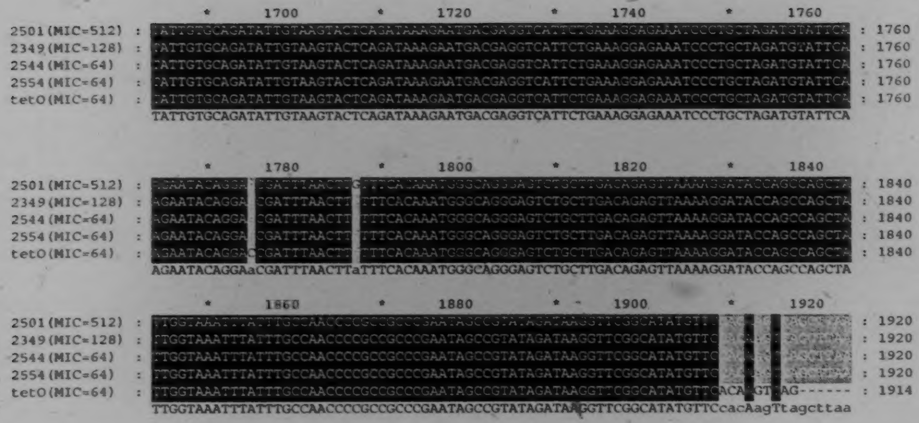



```
                     *     1700       *      1720       *      1740       *      1760
2501(MIC=512) : TATTGTGCAGATATTGTAAGTACTCAGATAAAGAATGACGAGGTCATTCTGAAAGGAGAAATCCCTGCTAGATGTATTCA : 1760
2349(MIC=128) : TATTGTGCAGATATTGTAAGTACTCAGATAAAGAATGACGAGGTCATTCTGAAAGGAGAAATCCCTGCTAGATGTATTCA : 1760
2544(MIC=64)  : TATTGTGCAGATATTGTAAGTACTCAGATAAAGAATGACGAGGTCATTCTGAAAGGAGAAATCCCTGCTAGATGTATTCA : 1760
2554(MIC=64)  : TATTGTGCAGATATTGTAAGTACTCAGATAAAGAATGACGAGGTCATTCTGAAAGGAGAAATCCCTGCTAGATGTATTCA : 1760
tetO(MIC=64)  : TATTGTGCAGATATTGTAAGTACTCAGATAAAGAATGACGAGGTCATTCTGAAAGGAGAAATCCCTGCTAGATGTATTCA : 1760
                TATTGTGCAGATATTGTAAGTACTCAGATAAAGAATGACGAGGTCATTCTGAAAGGAGAAATCCCTGCTAGATGTATTCA

                     *     1780       *      1800       *      1820       *      1840
2501(MIC=512) : AGAATACAGGA CGATTTAACTTGTTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGGATACCAGCCAGCTA : 1840
2349(MIC=128) : AGAATACAGGA CGATTTAACTT TTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGGATACCAGCCAGCTA : 1840
2544(MIC=64)  : AGAATACAGGA CGATTTAACTT TTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGGATACCAGCCAGCTA : 1840
2554(MIC=64)  : AGAATACAGGA CGATTTAACTT TTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGGATACCAGCCAGCTA : 1840
tetO(MIC=64)  : AGAATACAGGACCGATTTAACTT TTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGGATACCAGCCAGCTA : 1840
                AGAATACAGGAaCGATTTAACTTaTTTCACAAATGGGCAGGGAGTCTGCTTGACAGAGTTAAAAGGATACCAGCCAGCTA

                     *     1860       *      1880       *      1900       *      1920
2501(MIC=512) : TTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGGCATATGTTC[illegible] : 1920
2349(MIC=128) : TTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGGCATATGTTC[illegible] : 1920
2544(MIC=64)  : TTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGGCATATGTTC[illegible] : 1920
2554(MIC=64)  : TTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGGCATATGTTC[illegible] : 1920
tetO(MIC=64)  : TTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGGCATATGTTCACAAGTTAG------ : 1914
                TTGGTAAATTTATTTGCCAACCCCGCCGCCCGAATAGCCGTATAGATAAGGTTCGGCATATGTTCcacAagTtagcttaa
```

Analysis of the nucleotide substitutions in *C. jejuni* clinical isolate 25-01 did not identify any major changes of the amino acid sequences of the *tet*(*O*) proteins (Table 2.8). In six of the seven positions, although changes in the amino acid sequence were predicted, they did not explain the increased MIC. For example, a mutation at nucleotide position 993 changed an isoleucine to a methionine. Both of these, however, are non-polar amino acids. In one instance, a mutation at nucleotide position 910 resulted in a change from a serine to a proline, which is a change from a polar amino acid with a hydroxyl side chain to a cyclic, non-polar amino acid. Sequence data on the regions of interest in 25-01 was also obtained for two other high-level tetracycline-resistant isolates (23-51 and 16-42). Only one nucleotide substitution in *tet*(*O*) (at position 993) was present in all three sequences. The potential effect of these changes on the Tet(O) protein is unknown. Therefore, no obvious explanation for the increased MIC in *C. jejuni* isolates by these *tet*(*O*) genes was identified.

2.3.7 Cloning of a *tet*(*O*) gene into the pRY expression vectors

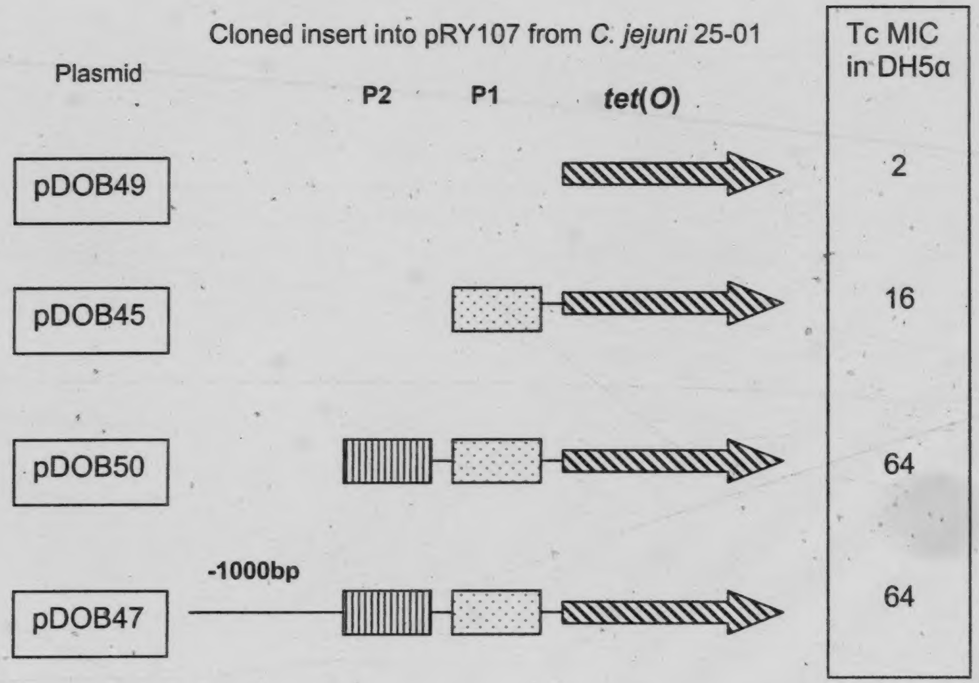
The upstream region of *tet*(*O*) is known to be important for full expression of tetracycline resistance (169). To determine if the upstream region of the *tet*(*O*) gene plays a role in mediating extremely high levels of tetracycline resistance, the *tet*(*O*) determinant was cloned from *C. jejuni* clinical isolate 25-01 (tetracycline MIC=512 μg/mL) incorporating increasing portions of its upstream region. As presented in Figure 2.6, the P2 promoter of the *tet*(*O*) gene was found to be important in mediating tetracycline resistance in *E. coli*.

Table 2.8. DNA sequence analysis of the *tet*(*O*) gene (1.92 kb) cloned from tetracycline-resistant *C. jejuni* clinical isolate (25-01) from the 512 μg/mL MIC level.

	DNA sequence		**Amino acid sequence**			**25-01 sequence change present in Tc^{r} *C. jejuni* 512 µg/mL 16-42 and 23-51**	
Nucleotide position in *tet*(*O*) ORF (1.92 kb)	**GenBank *tet*(*O*)**	***tet*(*O*) Tc^{r} 512 µg/mL *C. jejuni* (25-01)**	**GenBank Tet(O)**	**Tet(O) Tc^{r} 512 µg/mL *C. jejuni* (25-01)**	**Effect of mutation on amino acid properties**	**16-42**	**23-51**
884	A	G	Y	C	Both uncharged polar	YES	NO
910	T	C	S	P	Polar with hydroxyl side chain to cyclic non polar	YES	NO
993	A	G	I	M	Both non polar	YES	YES
1036	A	C	I	L	Both non polar	N/A	N/A
1063	T	C	S	P	Uncharged polar to non polar	YES	NO
1111	T	G	C	G	Uncharged polar to non polar	YES	N/A
1772[a]	C	A	T	N	Both uncharged polar	YES	YES
1784	A	G	Y	C	Both uncharged polar	N/A	N/A

[a] Mutation at nucleotide position 1772 is shaded in grey, which is found in all *tet*(*O*) sequences in a number of studies, indicating a mistake in the GenBank sequence.

Figure 2.6. Cloning of the *tet*(*O*) gene and segments of its upstream region into *C. jejuni*-*E. coli* shuttle-expression vector pRY107. Primer combinations are listed (5' and 3', sequences in Table 2.2. The *tet*(*O*) gene is designated by an arrow with black diagonal lines, promoters P1 and P2 by dotted and lined boxes, respectively, and a line designates an undefined upstream region (-1000 bp). Tetracycline (Tc) MIC is in μg/mL.


Cloned insert into pRY107 from *C. jejuni* 25-01
Plasmid
P2
P1
tet(*O*)
Tc MIC in DH5α
pDOB49
2
pDOB45
16
pDOB50
64
-1000bp
pDOB47
64

The inclusion of the P2 promoter with *tet*(*O*) in the pRY107 expression vector was found to mediate higher levels of tetracycline resistance (64 μg/mL) than constructs containing only P1 or the *tet*(*O*) ORF only (16 and 2 μg/mL, respectively). Unfortunately, electroporation and transformation of these vectors into a tetracycline-susceptible strain of *C. jejuni* were unsuccessful and the importance of the upstream region of the *tet*(*O*) gene for mediating high levels of tetracycline resistance in *C. jejuni* has not yet been resolved.

2.3.8 Attempts at insertional mutagenesis of *tet*(*O*)

To conclusively show that *tet*(*O*) is responsible for mediating the extremely high levels of tetracycline resistance in *C. jejuni*, the knockout of *tet*(*O*) through insertional mutagenesis was attempted. A "suicide vector" knockout strategy was attempted to knockout the *tet*(*O*) gene in the parental *C. jejuni* isolate 25-01 (tetracycline MIC=512 μg/mL) (163). A kanamycin-resistance cassette was inserted into an unique *Eco*47III site within the *tet*(*O*) gene cloned into pMS119EH (pDOB42). When transformed into *E. coli* DH5α, this vector did not confer tetracycline resistance (MIC<2 μg/mL).

E. coli expression vectors, such as pMS119EH, do not replicate in *C. jejuni*. However, electroporation or transformation of this construct (pDOB42) into *C. jejuni* isolate 25-01 should result in homologous recombination of the Km:*tet*(*O*) into the parent *tet*(*O*) gene. Thus while the plasmid does not replicate and will not be maintained in *C. jejuni*, gene knockouts can occur, as under selective pressure there can be recombination of the kanamycin cassette into the wild type *tet*(*O*) gene (163). Unfortunately, all attempts at electroporation or transformation of the pDOB42 suicide vector into isolate 25-01 were unsuccessful. Thus, it was not possible to determine if the high levels of tetracycline resistance were mediated solely by *tet*(*O*).

2.4 Discussion

2.4.1 Antibiotic resistance frequencies in *C. jejuni*

Increasing antibiotic resistance frequencies in *C. jejuni* are a great concern, as antibiotic treatment of severe infections may be rendered ineffective by resistant isolates. A study by Gaudreau and Gilbert (43) in Quebec, Canada has shown 53% of *C. jejuni* clinical isolates are tetracycline-resistant, up from only 19% in 1992-93. The work in this thesis has shown that in Alberta, Canada, the incidence of tetracycline resistance in *C. jejuni* has increased significantly to 49.8% (1999-2002) from 8.6% in 1981 (145). Thus, the considerable increase in the incidence of tetracycline resistance is a trend which is occurring across Canada. Globally, there is an increasing trend in the incidence of tetracycline resistance in *C. jejuni*. In Japan and the United States, 55% of clinical isolates are tetracycline-resistant (105, 126), and resistance reaches 95% in Taiwan (87).

A comparable rise in resistance to erythromycin and ciprofloxacin, the primary antibiotics used in the treatment of *C. jejuni* gastroenteritis, has not occurred in Alberta over the past twenty years. These results confirm other Canadian studies that have identified low levels of erythromycin resistance (43, 54). Low frequencies of erythromycin resistance have continued to occur in clinical isolates of *C. jejuni* in Alberta, Canada since the early 1980's (145). This is comforting news for clinicians, as erythromycin is the drug of choice for treating *C. jejuni* gastroenteritis and its efficacy has not been compromised by resistance. Similar results were found for ciprofloxacin, for which resistance was at only 2% (4 out of 203 isolates). In contrast, susceptibility data from other countries demonstrates that ciprofloxacin resistance in *C. jejuni* is emerging at a rapid rate, with some countries now reporting resistance levels of up to 30% (91). This may be due to the difference in antibiotics used to treat animals and as growth promotional agents (32).

In terms of the level of resistance to tetracycline, Taylor *et al.* (145) found that the tetracycline MICs varied between 32 and 128 μg/mL for tetracycline-resistant isolates in 1980-81. In 2000-2002, tetracycline MIC levels were higher, with 37% of isolates having MICs of 256-512 μg/mL tetracycline. Gaunt and Piddock (45) found an association between fluoroquinolone resistance and high tetracycline MICs but no such association was observed in this study.

Although antibiotic susceptibility testing for *C. jejuni* has yet to be standardized, determining MICs by the agar-dilution method remains the reference method (43). Unfortunately, MIC determination can vary because of inoculum size or growth (107). However the observed increase in the level of *tet*(*O*)-mediated tetracycline resistance to 512 μg/mL tetracycline is believed to be accurate, as MIC assays were performed in triplicate and control strains of

C. jejuni with known MICs to tetracycline were used throughout the experimental procedures.

2.4.2 The *tet*(*O*) gene: widespread occurrence in *C. jejuni*

Tetracycline resistance in *C. jejuni* is mainly mediated by plasmids (147). In this study, plasmid content was found to generally agree with previous surveys on *C. jejuni* clinical isolates. Plasmids were isolated from 50% of isolates, with 67% and 5% in tetracycline-resistant isolates and susceptible isolates, respectively. Previous studies have found plasmids in up to 82% of tetracycline-resistant isolates of *C. jejuni*, and in 15% of tetracycline-susceptible isolates (126). All tetracycline-resistant *C. jejuni* isolates in the 1980-81 Alberta study contained plasmids (145). Note that while all tetracycline-resistant isolates were screened for plasmids, not all susceptible isolates were included in the plasmid content survey.

The plasmid-encoded *tet*(*O*) gene is the primary tetracycline-resistance determinant in *C. jejuni*. PCR screens and DNA-DNA hybridizations showed that the *tet*(O) gene is widespread in *C. jejuni*, as it was found in all tetracycline-resistant clinical isolates. A PCR screen for the *tet*(*O*) tetracycline-resistance gene is effective for generating products from a number of templates, including the following: purified plasmid DNA, total DNA and whole-cell boiled preparations. This PCR screen was used to identify *tet*(*O*) in 94% of the tetracycline-resistant isolates and only in six cases was it not able to amplify a product from the boiled cell preparation. Mohran *et al*. (102) have suggested that a certain subset of the *C. jejuni* population is restrictive to releasing DNA by simple boiling. For these six isolates, DNA-DNA hybridizations were successful in identifying *tet*(*O*). Therefore, the *tet*(*O*) gene may either be located on a plasmid which was not able to be isolated or more likely, on the chromosome.

2.4.3 Cloning, sequencing and characterization of the *tet*(*O*) gene

Previous research has determined that the *tet*(*O*) gene can confer resistance to an MIC of 128 μg/mL tetracycline in *C. jejuni* (145). In this study, plasmids that harboured *tet*(*O*) were shown to mediate significantly higher levels of resistance to tetracycline (512 μg/mL) in four of the 101 tetracycline-resistant isolates. This was demonstrated by the conjugative transfer of these plasmids to tetracycline-susceptible recipient strains of *C. jejuni*, in which the transconjugants had tetracycline MICs identical to the donor strains (512 μg/mL).

Cloning of the *tet*(*O*) gene from plasmids encoding a range of MIC levels (64 – 512 μg/mL tetracycline) into *E. coli* DH5α (using pMS119EH as a vector) resulted in tetracycline resistance of 32 μg/mL. Thus, the *tet*(*O*) ORF confers a lower level of tetracycline resistance, regardless of the MIC it conferred in *C. jejuni*. Taylor *et al.* (143) found no barrier to expression of *tet*(*O*), as the MIC of *C. jejuni* UA466 (64 μg/mL), was the same as the MIC for *tet*(*O*) cloned into *E. coli* JM105.

The upstream region of the *tet*(*O*) gene is important for full expression of tetracycline resistance (169) and therefore may play a role in the high levels of tetracycline resistance in the four isolates with MICs of 512 μg/mL. In their analysis of the sequenced region (GenBank accession number M74450), Wang and Taylor assigned promoters P1 and P2 to *C. coli* and *E. coli*, respectively (169). Promoter P1 had only five of twelve nucleotides in common with the *E. coli* promoter consensus sequences and transcription from P1 was poor in *E. coli*, conferring only a low level of tetracycline resistance. As the same plasmid mediated high levels of resistance to tetracycline in *C.coli*, P1 was designated as a *Campylobacter tet*(*O*) promoter (169). The other potential promoter sequence upstream of *tet*(*O*), P2, was at -281 upstream of the ATG start codon and had 100% identity for its -10 and -35 regions with the *E. coli* promoter consensus sequences (169). Transcription from P2 was demonstrated in *E. coli* but not in *C. coli*. Wang and Taylor (169) concluded that the region upstream of *tet*(*O*) (300 bp) is essential for full expression of tetracycline resistance in *E. coli*, but its function remains unknown.

Expression of the *tet*(*O*) gene from pRY107 or pRY108 in *E.coli* DH5α resulted in MICs that were dependent on the presence of the P2 promoter. Further study of *tet*(*O*) expression in *C. jejuni* was not possible, as electroporation or transformation of *tet*(*O*) cloned into pRY107 or pRY108 into tetracycline-susceptible strains of *C. jejuni* was uniformly unsuccessful. Thus, the importance of the *tet*(*O*) promoter region in mediating high levels of tetracycline resistance in *C. jejuni* was not resolved.

The *tet(O)* genes from high-level tetracycline-resistant *C. jejuni* isolates (MICs of 512 μg/mL) were shown to differ in seven distinct nucleotide positions from the *tet*(*O*) gene from isolates with lower MICs (64-128 µg/mL). The effect of these mutations on the Tet(O) protein is unknown and no crystal structure of Tet(O) is presently available. However, residue substitutions were compared to the structure of EF-G (from *Thermus thermophilus*), described in Chapter 1, which shares high sequence similarity to Tet(O). None of the substitutions are believed to be in important functional regions of Tet(O), although little is known about the functional hot spots in Tet(O), apart from the GTP binding motifs (S. Connell, personal communication). Analysis of substituted residue locations on Tet(O) (using the EF-G structure) has identified that several are exposed on the surface of the protein and may be involved with interacting with the ribosome. These mutations may potentially allow for stronger binding to the ribosome and therefore allow Tet(O) to out-compete high levels of tetracycline.

Differences in MICs among lower level tetracycline-resistant isolates (32-128 µg/mL) may be due to host differences. It is possible that a plasmid-mediated efflux system is acting in the high-level tetracycline-resistant isolates. Alternatively, the pTet plasmids may also upregulate an intrinsic efflux system. The *cme*ABC antibiotic efflux pump is present in *C. jejuni* (88), and screening for these genes in the four high-level tetracycline-resistant isolates may be worthwhile in future research. Another potential experiment to identify a potentially novel *tet* gene would be to perform a restriction digest on the pTet plasmids, clone the various segments into an *E. coli* vector and select for tetracycline resistance after transformation of a tetracycline-susceptible *E. coli* strain.

Tetracycline-resistance plasmids (pTet) from *C. jejuni* have been sequenced and appear to be highly conserved over decades, as a plasmid isolated in the late 1970's was virtually identical in its DNA sequence to a plasmid isolated in 2000 (P. Guerry, personal communication). It is unknown if these plasmids encoded novel *tet* genes. It is not common for facultative Gram-negative enteric bacteria to carry multiple types of *tet* genes (19) and therefore it is unlikely that the extremely high levels of resistance in *C. jejuni* are due to multiple *tet* determinants. However, the up-regulation of an intrinsic efflux system is a possibility that cannot be excluded.

The study of the *tet*(*O*) genes from high-level tetracycline-resistant isolates would have been strengthened by the expression of the *tet*(*O*) gene in a tetracycline-susceptible strain of *C. jejuni*. For this reason, the *tet*(*O*) determinant from isolate 25-01 was cloned into the pRY107 and pRY108 *C. jejuni-E. coli* shuttle-expression vectors (179). Unfortunately, the electroporation and transformation of these vectors into *C. jejuni* was not successful. Future research in *C. jejuni* is hampered by a lack of basic genetic engineering techniques.

Campylobacter sp. have been shown to be naturally transformable (170) but not all *C. jejuni* strains are competent. There is substantial variation in the ability of *C. jejuni* strains to be transformed by plasmid DNA (171), with *C. jejuni* displaying a strong preference for its own DNA (170). Takata *et al.* (139) found that *C. jejuni* NCTC 11168 and 81-176 were not electro-competent for pRY111, a vector similar to pRY107 and pRY108.

Another method for introducing the expression vectors into *C. jejuni* would be to use the mobilizing donor strain S17-1, which contains a chromosomally integrated IncP plasmid (RP4) and can be used to transfer vectors with a P-type mobilization site (Mob) between Gram-negative bacteria (132). As Simon *et al.* (132) note, S17-1 is useful in two respects: 1) it does not contain any *E. coli* K12 DNA restriction enzymes, so that any transformed foreign DNA is taken up efficiently, and 2) it is kanamycin-resistant, which allows for selection of *Tn5* containing plasmids.

2.5 Conclusions and Future Directions

This study supports a dramatic increase in the prevalence of tetracycline resistance in human clinical isolates of *C. jejuni* in Alberta, Canada over the last two decades. A similar increase in the prevalence of erythromycin and ciprofloxacin, antibiotics often used to treat or prevent *C. jejuni* infections, has not occurred. Continued surveillance of antibiotic resistance among clinical isolates would establish whether or not the increasing trend in tetracycline resistance will continue. Furthermore, future surveillance would also determine if fluoroquinolone resistance will emerge in Alberta, as it has in many nations worldwide.

An analysis of resistance rates among animal isolates would provide critical data in identifying the source of increasing tetracycline-resistance rates. Such a study should include isolates from poultry, the primary source of human

C. jejuni infections. Also analyzing isolates from other animal sources, such as cattle and wild birds, would provide further interesting epidemiological data.

Although this study has identified that a subset of *C. jejuni* strains are resistant to very high levels of tetracycline, it has not resolved the molecular basis of the resistance. Techniques for the genetic manipulation of *Campylobacter* need to be improved, particularly for transformation and electroporation procedures. Continued monitoring of MIC levels in tetracycline-resistant isolates of *C. jejuni* should be undertaken to determine if resistance levels continue to increase. It is also critical for researchers in the *Campylobacter* field to agree on standardized antibiotic susceptibility testing methods, as considerable variability exists in the reporting of results and the execution of methods in the literature.

Chapter 3

Virulence plasmid pVir and its association with clinical outcome of *C. jejuni* infection

3 Virulence plasmid pVir and its association with clinical outcome of *C. jejuni* infection

3.1 Introduction

The food-borne enteric pathogen, *Campylobacter jejuni*, is a leading cause of bacterial gastroenteritis (10, 99). Infection with *C. jejuni* may result in diarrhea, fever, abdominal pain, vomiting, and the presence of blood in stool, the latter being an indicator of a severe invasive infection (133). In addition, serious sequelae such as Guillian-Barré syndrome and bacteremia may also occur with *C. jejuni* infections (52, 106).

Various virulence factors have been proposed to contribute to the pathogenesis of *C. jejuni*, which allow for adherence, colonization and invasion of the intestinal epithelium (61). Potential virulence components include flagella (48), invasion proteins (Cia proteins) (73) and toxins (CDT) (174). Although its genome has been sequenced (113) and substantial research has been performed on its putative virulence factors, the mechanisms of *C. jejuni* pathogenicity remain poorly understood (162).

A number of bacterial enteric pathogens contain plasmids which contribute to pathogenesis, including *Shigella* sp. (129), *Salmonella* sp. (50, 51), and enteropathogenic *Escherichia coli* (155). In *C. jejuni*, plasmids were reportedly present in up to 52% of human clinical isolates (126) and have been found to primarily mediate antibiotic resistance (145). One such plasmid encodes the tetracycline-resistance determinant *tet*(O) (named pTet by (5)) and which has been transferred between strains of *Campylobacter* sp. but not to *E.*

coli, suggesting its host range is restricted (146). The size of the pTet plasmid is approximately 37 kb (5).

There was no evidence for the involvement of plasmids in the virulence of *C. jejuni* until Bacon *et al.* (5) identified plasmid pVir in strain 81-176, which was originally isolated from an outbreak of *C. jejuni* gastroenteritis from unpasteurized milk (76). This ~37.5 kb plasmid contains components of a T4SS (6), which is known to be important for the virulence of a number of major bacterial pathogens, including *Bordetella pertussis*, *Legionella pneumophilia* and *Helicobacter pylori* (20).

In their initial study, Bacon *et al.* (5) presented evidence that pVir is important *in vitro* for both adherence and invasion of intestinal epithelial cells in culture. They also reported *in vivo* data, in which a mutation in pVir gene *cjp5* (a *virB11* homolog) reduced the virulence of *C. jejuni* 81-176 in the ferret disease model. The *virB11*-type genes encode nucleotide-binding proteins that are proposed to provide the energy for secretion of macromolecules (20). In a survey of fresh clinical isolates of *C. jejuni*, 10% (*n*=58) contained pVir (5), based upon DNA-DNA hybridizations of colony blots with a ^{32}P-labelled *cjp5* (*virB11*) DNA probe.

Virulence in enteric pathogens may also be associated with antimicrobial resistance, including increased hospitalization periods and disease complications in patients infected with antibiotic-resistant strains (156). For example, fluoroquinolone resistance has been linked to prolonged duration of clinical symptoms in *C. jejuni* infections, regardless of whether or not the patient was treated with ciprofloxacin (94). The National Antimicrobial Resistance Monitoring System (NARMS) reported that untreated patients with ciprofloxacin-resistant infections had longer periods of diarrhea than those with ciprofloxacin-susceptible infections (96). These observed effects may be due to the increased virulence in ciprofloxacin-resistant *C. jejuni* (156).

This chapter considers the clinical outcomes of infection with *C. jejuni*, in relation to the presence of the pVir virulence plasmid and the occurrence of antibiotic resistance. The primary objective was to determine if the presence of the pVir virulence plasmid correlates with severe symptoms in *C. jejuni* clinical infections. Plasmid content and antibiotic-resistance frequencies were analyzed in 137 *C. jejuni* clinical isolates, obtained from patients with campylobacteriosis between 1999 and 2002 from the APLPH. Virulence plasmid incidence and antibiotic resistance were compared to clinical symptoms collected from medical records and patient questionnaires from 105 patients. Clinical data was collected by Dr. J. Ahmed-Bentley, under the supervision of Dr. K. Kowalewska-Grochowska. The presence of blood in patient stool occurred at a higher frequency in patients infected with a *C. jejuni* strain that carried pVir. Furthermore, pVir was found primarily in *C. jejuni* strains carrying the tetracycline-resistance plasmid, pTet, but unlike the latter, was not able to transfer to other strains of *C. jejuni*.

3.2 Materials and Methods

3.2.1 *C. jejuni* strains

Clinical isolates of *C. jejuni* were obtained from the APLPH, as described in Chapter 2, Materials and Methods. A total of 105 isolates to which clinical data could be matched were included in the study sample for pVir incidence and clinical outcomes. All major strains used in this study are listed in Table 3.1. In the study of pVir and pTet co-incidence, 137 isolates were analyzed. Although many of the 105 isolates with clinical data were included among the 137 isolates in the pVir-pTet co-incidence study, the overlap is not perfect. Furthermore, a large proportion of these 137 isolates were included in the antibiotic resistance study (Chapter 2), however the strain populations are not identical.

3.2.2 Antibiotic resistance

Antibiotic disc diffusion was used to determine the antibiotic susceptibility of all 137 isolates in this study, as described in Chapter 2, Materials and Methods. Briefly, each isolate was tested for susceptibility to 5 antibiotic discs (Oxoid): tetracycline (30 μg), kanamycin (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), nalidixic acid (30 μg). Nalidixic acid-resistant strains were also tested for resistance to ciprofloxacin (1 μg).

Table 3.1. Strains and plasmids used in this study.

Strain or plasmid	Relevant Characteristics	Selective Marker(s)[a]	Reference or Source
E. coli			
DH5α		Nal	Invitrogen
C. jejuni			
UA 543		Nal	
81-176		Tc	(5)
***C. jejuni* clinical isolates**			
23-51		Tc (MIC=512μg/mL)	APLPH
Other isolates		Various	APLPH
Plasmids			
pVir	Putative virulence plasmid from *C. jejuni* 81-176		(5)
pMS119EH		Amp	(137)
R27		Tc	(131)

[a] Tc, tetracycline; Nal, nalidixic acid; Amp, ampicillin.

3.2.3 Plasmid isolation

For the *C. jejuni* isolates with matched clinical data, plasmids were isolated from both frozen stocked cultures of *C. jejuni* (*n*=78), maintained in skim milk or BHI (Difco) supplemented with 20% glycerol, or from fresh clinical isolates (*n*=27). Plasmid isolations were performed using a Qiagen Midi or Mini Plasmid Kit (Qiagen), by the alkaline lysis method (127) or by a modified method used for *H. pylori* (30), as described in Chapter 2, Materials and Methods.

3.2.4 DNA-DNA hybridizations for pVir

3.2.4.1 Application and fixation of DNA to nitrocellulose paper

Purified plasmid DNA (up to 200 μg DNA) was pipetted onto nitrocellulose paper and allowed to dry. The applications included purified plasmid DNA from the *C. jejuni* clinical isolates, *C. jejuni* 81-176 (positive control for pVir) (5) and plasmids R27 (131) and pMS119EH (negative controls). The location of each plasmid sample was recorded and the orientation of the nitrocellulose paper marked by cutting off the top right corner. Plasmid DNA on the nitrocellulose paper was denatured in Solution 1 (0.5 M NaOH, 0.15 M NaCl) (127) for 5 minutes on a shaker. Nitrocellulose paper was blotted dry on a towel to remove excess liquid. Neutralization was performed in Solution 2 (10 mM Tris HCl, 0.15 M NaCl, pH 7.5) (127) for 5 minutes, blotted to dry and repeated. Nitrocellulose paper was then soaked in 2x SSPE solution (127) for 5 minutes and dried. All washes were performed in a plastic tray containing a piece of chromatography paper (FisherBrand, Pittsburgh, PA). DNA was baked onto the nitrocellulose, covered in chromatography paper, in a drying oven at 50°C overnight (approximately 18 hours) and at 65°C for three hours on the following day.

3.2.4.2 Preparation of *cjp5/virB11* DNA probe

Primers DMT 3 and DMT 4 (Table 3.2) were used to PCR amplify a 708 base pair product from within the *cjp5* gene on pVir (5). The conditions for the PCR reaction were as follows: initial denaturation at 95°C for 1 minute, followed by 30 cycles of denaturation (1 minute, 95°C), annealing of primers (1 minute, 50°C) and primer extension (1 minute, 72°C). PCR was performed in a BioRad Gene Cycler (BioRad).

The *cjp5* PCR product was purified by a PCR clean up kit (Qiagen) and eluted with TE buffer. An aliquot of 225 ng of the *cjp5* PCR product was diluted with sterile Milli-Q water to a final volume of 20 μL and was placed into a 1.5 mL microfuge tube. DNA was denatured by boiling for 5 minutes and the tube was placed directly on ice after boiling.

A random primers DNA labeling system (Gibco) was used to prepare the ^{32}P-labelled *cjp5* DNA probe. On ice, 2 μl of each of dATP, dGTP and dTTP solutions were added to the DNA and mixed, followed by the addition of 15 μl of random primers buffer mixture. 5 μl of [α-^{32}P] dCTP was added following radioactive materials safety procedures. Sterile water was added (3 μl) to a

Table 3.2. Primers used in this study.

Primer	Sequence	Purpose
DMT 3	5' GAACAGGAAGTGGAAAAACTAGC 3'	Amplification of a 708bp fragment of *cjp5* (*virB11*) (5' end)
DMT 4	5' TTCCGCATTGGGCTATATG 3'	Amplification of a 708bp fragment of *cjp5* (*virB11*) (3' end)

total volume of 49 µl. The solution was mixed briefly by tapping the microfuge tube. 1 µl of Klenow fragment was added to the mixture, which was mixed gently and the tube centrifuged briefly. Mixture was incubated at 25°C for one hour behind a radiation shield and then boiled for 5 minutes to denature the DNA probe. The ^{32}P-labeled *cjp5* DNA probe was used immediately for DNA-DNA hybridization experiments to screen isolated *C. jejuni* plasmids for the presence of pVir, as described in (5).

3.2.4.3 Preparation of the hybridization solution

Denhart solution [0.2% Ficoll (Sigma), 0.2% polyvinyl pyrrolidone (PVP, Sigma), 0.2% bovine serum albumin (BSA; Sigma) in filter sterilized Milli-Q water] was prepared fresh before each hybridization. The hybridization solution consisted of 6x SSC (127), 5x Denhart solution, 0.1% sodium dodecyl sulfate (SDS; BioRad) and 100 µg/mL herring sperm DNA (Invitrogen), the latter being added immediately before hybridization.

3.2.4.4 Hybridizations

The nitrocellulose paper, inoculated with denatured and fixed plasmid DNA, was wetted with 2x SSC (127) solution in a hybridization tube and mixed for 5 minutes at 50°C in a Micro4 incubation chamber (Hybaid, Ashford, UK) on a gentle rotation cycle. SSC solution was discarded and the hybridization solution was added to the tube and mixed for 2 hours in the chamber at 50°C. The boiled, ^{32}P-labeled *cjp5* DNA probe was added directly to the hybridization solution in the tube, mixed, and incubated overnight (approximately 18 hours) at 50°C.

Radioactive hybridization solution was discarded into the radioactive waste containers and the probed nitrocellulose paper was washed in 0.5x SSC solution, removed from the hybridization tube and dried between sheets of chromatography paper. The radioactive nitrocellulose paper was then covered in plastic wrap and placed into a developing cartridge and exposed to BioMax MS Film (Eastman Kodak Company, Rochester, NY) overnight at -70°C. Film was developed in a Kodak developing machine and the image scanned into Corel Photopaint 10 (Corel Corporation).

3.2.5 Transfer of plasmids between *C. jejuni* strains

Transfer of plasmids from *C. jejuni* clinical isolate 23-51 to *C. jejuni* UA 543 (a plasmid-less, nalidixic acid-resistant, tetracycline-susceptible recipient strain), was performed by the method of Taylor *et al.* (146). Suspensions of donor strain 23-51 and UA 543 recipient strains were mixed in ratios of 1:4 and 1:6, centrifuged and resuspended in 100 µL of LB broth (Difco). Mating suspensions were inoculated onto a 0.22 µm Millipore filter (Millipore Corporation, Nepean, ON) on BHI agar and incubated for 24 hours. Cultures were resuspended in 1 mL of PBS, diluted, and plated onto MH agar containing 25 µg/mL tetracycline. Plasmids were isolated from the transconjugants by a Qiagen Mini Kit. Plasmid sizes were confirmed by agarose gel electrophoresis. The *tet*(*O*) PCR screen was performed on isolated plasmid DNA as described in

Chapter 2, Materials and Methods. The *cjp5* DNA probe was used to screen for the pVir virulence plasmid as described above.

3.2.6 Collection of clinical data

Patient clinical data was collected by Dr. J. Ahmed-Bentley, under the supervision of Dr. K. Kowalewska-Grochowska. Data was obtained for 105 patients with *C. jejuni* gastroenteritis, which included information on symptoms (diarrhea, bloody diarrhea, pain, fever, vomiting), duration of symptoms, hospitalization (if required), antibiotic use, travel history and complications. Clinical data were obtained directly from patient medical charts and physician questionnaires.

3.2.7 Statistical analysis

Incidence of pVir and pTet plasmids was compared using the Chi square goodness of fit and a Fisher exact test. Both pVir incidence and antibiotic susceptibility were compared to clinical symptom occurrence by the Fisher exact test. Statistical significance was set to $p<0.05$. Duration of clinical symptoms was compared with the incidence of pVir and with the occurrence of antibiotic resistance, by a Mann-Whitney rank sum test using SigmaStat (Version 2.0, Jandel Corporation, San Rafael, CA).

3.3 Results

3.3.1 Antibiotic resistance

Antibiotic disc diffusion identified tetracycline resistance (to a 30 μg disc) in 78 of 137 isolates (57%) and was found in all isolates resistant to multiple antibiotics (>2 antibiotics). Nalidixic acid resistance (30 μg) was identified in 5 isolates (3.6%) and 4 of these were also ciprofloxacin resistant (2.9%). Kanamycin resistance (30 μg) was identified in 3 isolates (2.2%).

3.3.2 Clinical data on *C. jejuni* infections

In the cases with clinical data, the most commonly reported clinical symptom was diarrhea (98%), followed by pain (83%) and fever (64%). Bloody stool was reported in 27% of cases, just slightly less frequent than vomiting (30%). Hospitalization for treatment of severe dehydration occurred in 18% of cases and the estimated mean duration of clinical symptoms was 6.1 days.

3.3.3 Plasmid content in clinical isolates of *C. jejuni*

Plasmids were successfully isolated from 43% (59 of 137) of the clinical isolates. The absence of a plasmid in a particular isolate was confirmed by using several isolation methods in multiple attempts. The majority of plasmids isolated were ~35-40 kb plasmids (pTet) that mediated resistance to tetracycline through the *tet*(*O*) gene. pTet was found in 58 of 137 (42%) of isolates and in 58 of 78 (74%) of tetracycline-resistant isolates. The incidence and transfer of tetracycline- resistance plasmids, as well as the cloning of *tet*(O) has been described in Chapter 2.

The pVir virulence plasmid has previously been identified through DNA-DNA hybridizations on colony blots of *C. jejuni* clinical isolates (5). In this study, only purified plasmid DNA was screened by DNA-DNA hybridizations for the *cjp5* (a *virB11* homolog) gene on pVir. A ^{32}P-labelled *cjp5* probe was successful in identifying pVir from purified plasmid DNA preparations from *C. jejuni* clinical isolates (Figure 3.1). Studies using the *cjp5* probe indicated that 21 of 137 (15%) of *C. jejuni* clinical isolates contained pVir. pVir was present in 18 of the 105 isolates (17%) to which clinical data could be matched. Frozen stock isolates were found to have a higher incidence of pVir than fresh clinical isolates (23% to 11%, respectively).

Comparison of pVir and tetracycline resistance revealed 95% (*n*=21) of the pVir-containing *C. jejuni* isolates were resistant to at least 30 μg of tetracycline in the disc diffusion method. Similar results were found for the occurrence of pVir and pTet, as 20 out of 21 pVir positive isolates also contained pTet (Table 3.3). Thus only one isolate contained pVir only. Statistical analysis confirmed pVir was found largely in concert with tetracycline-resistance plasmids ($p<1 \times 10^{-8}$, Fisher exact test; $\alpha<0.001$, Chi square analysis) and with tetracycline-resistance ($p=0.000042$, Fisher exact test; $\alpha<0.0001$, Chi square analysis). In contrast, 38 out of 58 (66%) isolates that contained pTet did not contain pVir (Table 3.3), demonstrating that pTet-mediated tetracycline resistance does not occur exclusively with pVir.

Figure 3.1 Identification of virulence plasmid pVir using a ^{32}P-labelled *cjp*5 DNA probe on purified plasmid DNA (as described in Materials and Methods). Row 1: pVir (Control from *C. jejuni* 81-176), pMS119EH (Negative plasmid control), R27 (Negative plasmid control) and Row 2: a variety of clinical isolates identified by a either a number (ie. 23-51) or a letter-number code (ie. FI-1).

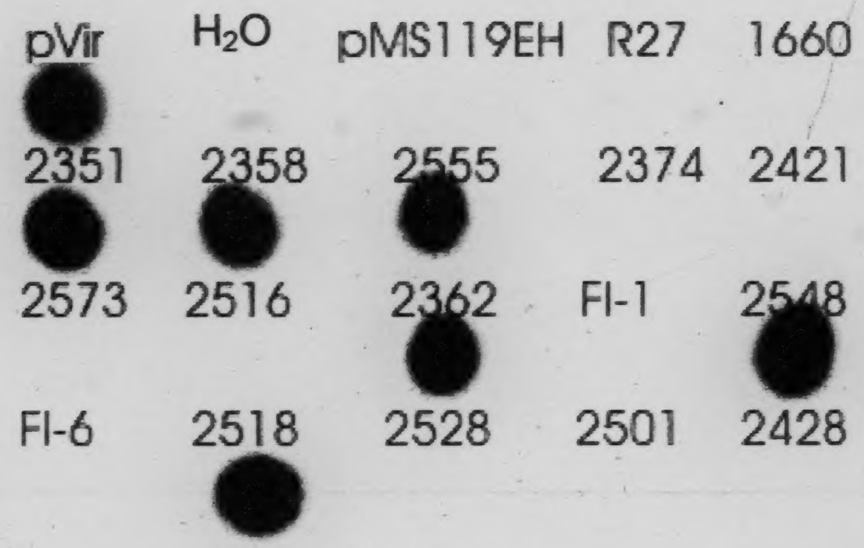

pVir
H₂O
pMS119EH
R27
1660
2351
2358
2555
2374
2421
2573
2516
2362
FI-1
2548
FI-6
2518
2528
2501
2428

Table 3.3. Incidence of pVir and pTet plasmids in *C. jejuni* and bloody stool in related infections. (A) Comparison of pVir and pTet plasmid content (*n*=137). (B) Comparison of pVir incidence and the occurrence of bloody stool in *C. jejuni* infections (*n*=93). (C). Comparison of bloody stool and antibiotic resistance among *C. jejuni* (*n*=93).

(A).

	pTet +	**pTet -**
pVir +	20	1
pVir -	38	78

(B).

	Bloody stool +	**Bloody stool -**
pVir +	9	8
pVir -	16	60

(C).

	Bloody stool +	**Bloody stool -**
Resistant	15	42
Susceptible	10	26

Isolate 23-51 contained both pVir and pTet and was resistant to extremely high levels of tetracycline (with an MIC of 512 µg/mL, as determined by the agar dilution method in Chapter 2). Bacon *et al.* (5) demonstrated that *C. jejuni* 81-176 contained both pTet and pVir, which have almost identical sizes, and demonstrated the loss of the pTet plasmid by using restriction digests on isolated plasmid DNA. A restriction digest of plasmids purified from *C. jejuni* clinical isolate 23-51 identified the presence of both pVir and pTet (Figure 3.2) as having similar bands to those obtained by Bacon *et al.* (5). This shows that a representative clinical isolate containing pVir and pTet from this study has the identical plasmid content to the previously characterized reference strain, *C. jejuni* 81-176. Furthermore, restriction profiling may be used for comparing pVir and pTet content in *C. jejuni* clinical isolates.

3.3.4 PCR is of limited use for detection of pVir

In Chapter 2, a PCR screen was employed with great success in identifying *tet*(*O*) on plasmids in *C. jejuni*. The use of PCR to screen for pVir would be of great assistance in epidemiological studies on *C. jejuni* clinical isolates. Unfortunately, attempts to use PCR to amplify the *cjp5* gene from pVir-containing clinical isolates of *C. jejuni* were uniformly unsuccessful (Figure 3.3). Therefore, a segment of the *cjp5* gene (708 bp) was amplified by PCR from *C. jejuni* 81-176, the original pVir reference strain, using primers DMT 3 and DMT 4 (Table 3.1, also see Materials and Methods). While these primers successfully amplified the *cjp5* gene from purified plasmid DNA or boiled whole-

Figure 3.2. *Bgl*II restriction digests of plasmids isolated from *C. jejuni*. Lane A-1 is 1kb DNA ladder. Plasmids from clinical isolate 23-51 (A-2) and reference strain 81-176 (B-1) had identical *Bgl*II restriction patterns, indicating that both contained the pVir and pTet plasmids. A tetracycline susceptible does not have the pTet plasmid (B-2) (See (5) for description). Gel B has been re-reproduced with permission from the study by Bacon *et al*. (5).

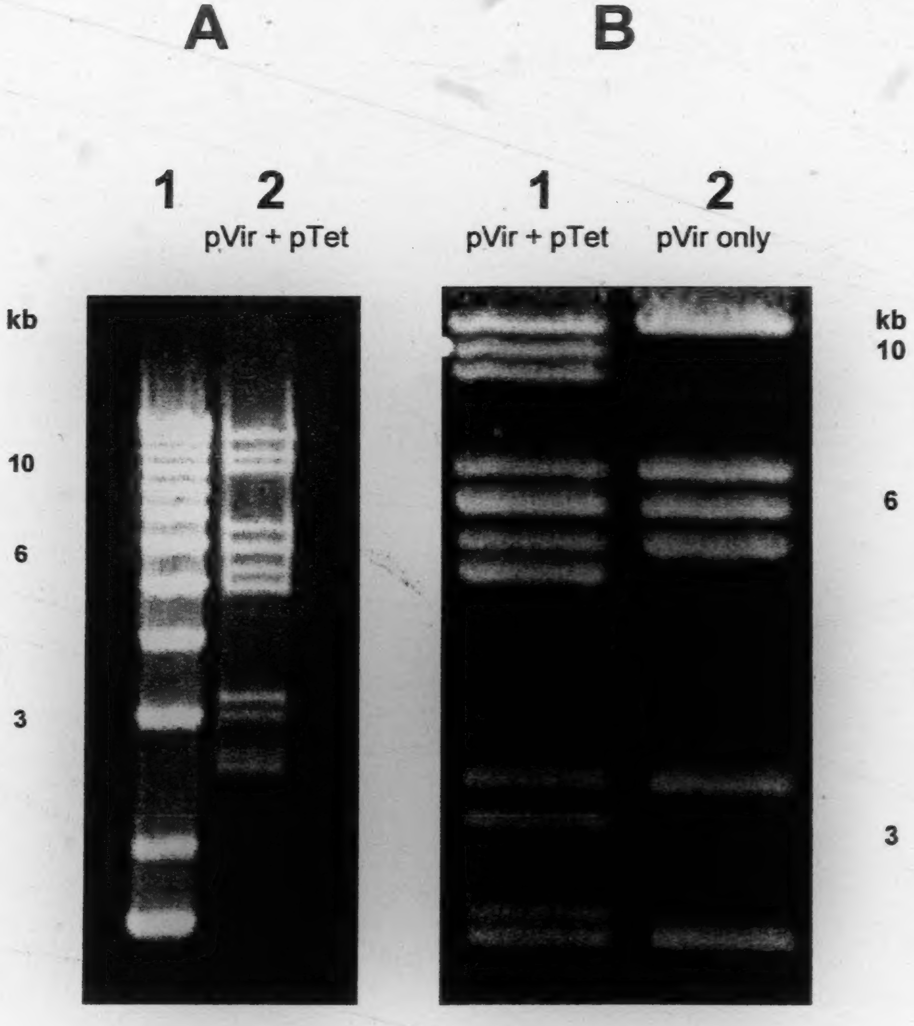
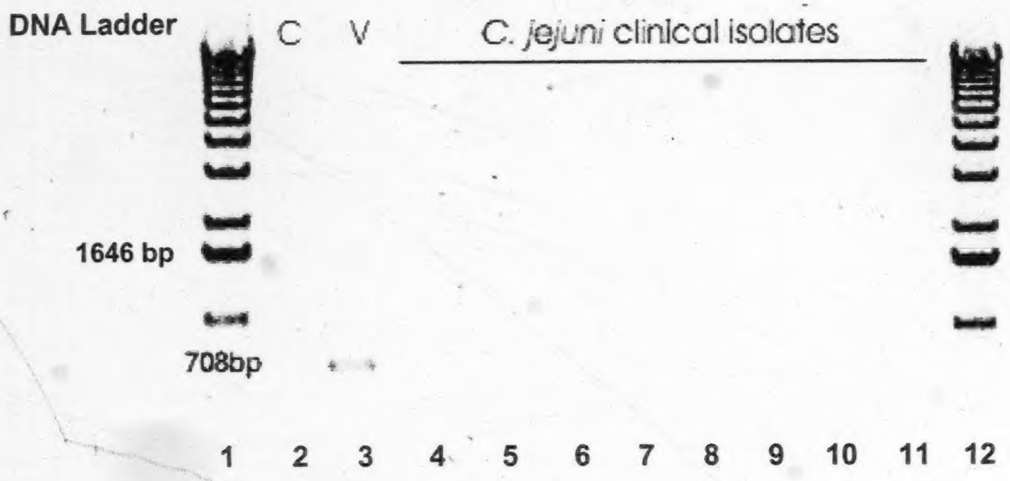

A
B
1
2
pVir + pTet
1
2
pVir + pTet
pVir only
kb
10
6
3
kb
10
6
3

Figure 3.3. PCR screen for pVir in *C. jejuni* clinical isolates. PCR amplification of a segment of the *cjp5* (*virB11*) gene (708 bp) was used to identify pVir in plasmid DNA (as described in Materials and Methods). PCR was not able to detect pVir in purified plasmid DNA or boiled whole cell preparations from clinical isolates of *C. jejuni*, even when pVir was identified through DNA-DNA hybridizations. Lanes 1, 12: 1 kb DNA ladder, Lane 2 (C, negative control reaction, pMS11EH plasmid DNA); Lane 3 (V, positive control, pVir isolated from 81-176); Lanes 4-11 (isolated plasmid DNA from various *C. jejuni* clinical isolates).


DNA Ladder
C
V
C. jejuni clinical isolates
1646 bp
708bp
1
2
3
4
5
6
7
8
9
10
11
12

cell preparations of *C. jejuni* 81-176, without a difference in product, no product was obtained from plasmid DNA, total DNA samples or from boiled whole-cell preparations of pVir-containing clinical isolates. Only DNA-DNA hybridizations with the radioactively labeled 708 bp *cjp5* PCR product successfully identified purified plasmid DNA for pVir (as described in Materials and Methods).

3.3.5 pVir and *C. jejuni* clinical infections

The association of the pVir virulence plasmid with symptom severity in human clinical infections with *C. jejuni* has not been reported previously. Statistical analysis of pVir content and clinical symptom occurrence determined that pain, diarrhea, vomiting and fever were equally likely to occur in infections with *C. jejuni*, regardless of the presence of pVir (Table 3.4). However, the presence of visible blood in patient stools, an indicator of severe invasive infections, was found to occur at a greater frequency in infections with a *C. jejuni* strain that contained pVir (p=0.011, Fisher exact test). Patients infected with pVir-containing *C. jejuni* strains had bloody stool in 50% of cases, compared to 18% in patients with non-pVir *C. jejuni* infections. Although pVir was associated with a greater occurrence of bloody stool, it was not a completely direct correlation, as some patients infected with non-pVir infections still reported bloody stool (16 out of 76 cases; see Table 3.3). Nevertheless, the association was statistically significant, and suggests that pVir may be clinically relevant. This was the first demonstration of the *in vivo* importance of pVir in human clinical infections with *C. jejuni*.

Table 3.3. Presence of the pVir virulence plasmid and occurrence of clinical symptoms in patients with *C. jejuni* gastroenteritis in Alberta, Canada (1999-2002).

	Clinical symptom						
pVir	**Pain**	**Diarrhea**	**Vomit**	**Blood in Stool**	**Fever**	**Hospitalization**	**Duration (Average)**
Present (n=18)	83% (*n=18)*	100% (*n* =18)	28% (*n* =16)	53% (*n* =17)	55% (*n* =17)	22% (*n* =18)	6.0 days (*n* =18)
Absent (n=87)	69% (*n* =72)	96% (*n* =86)	25% (*n* =73)	18% (*n* =76)	48% (*n* =64)	17% (*n* =87)	6.1days (*n* =81)
Significance[a]	No	No	No	Yes	No	No	No

[a] Statistical significance ($p<0.05$) was calculated using a Fisher Exact Test.

3.3.6 Antibiotic resistance and clinical outcomes

Antibiotic-resistant enteric pathogens have previously been linked to infections with prolonged or more severe clinical symptoms compared with antibiotic-susceptible strains (156). To determine if a similar association occurs with *C. jejuni* infections in Alberta, Canada, antibiotic resistant isolates (which always included resistance to a 30 μg tetracycline antibiotic disc) were compared with clinical symptoms (Table 3.5). Although no difference was found for patients infected with resistant strains of *C. jejuni* in terms of pain, diarrhea or fever, antibiotic-resistant isolates were associated with a higher frequency of vomiting (*p*=0.01, Fisher exact test). Statistical analysis of the duration of clinical symptoms and the incidence of antibiotic resistance was performed with a Mann-Whitney rank sum test, as the data did not meet the requirements for normality in a student's t-test. Patients infected with antibiotic-resistant strains of *C. jejuni* had a longer duration of clinical symptoms than patients infected with antibiotic-susceptible strains (6.7 vs. 4.9 days, respectively, *p*=0.037).

3.3.7 pVir does not transfer with pTet to other *C. jejuni* strains

Bacon *et al.* (5) described difficulties in transferring pVir, which does not carry a selectable marker. Even with the insertion of a kanamycin tag into pVir, plasmid transfer was still uniformly unsuccessful (5). In contrast, the tetracycline-resistance plasmid pTet does transfer between strains of *C. jejuni*

Table 3.4. Antibiotic resistance of *C. jejuni* isolates and the occurrence of clinical symptoms in patients with *C. jejuni* gastroenteritis in Alberta, Canada (1999-2002).

	Clinical symptom						
Antibiotic resistance[a]	**Pain**	**Diarrhea**	**Vomit**	**Blood in Stool**	**Fever**	**Hospitalization**	**Duration (Average)**
Resistant (n=64)	78% (n=57)	95% (n=63)	40% (n=55)	26% (n=57)	52% (n=51)	17% (n=64)	6.7 days(n=60)
Susceptible (n=41)	63% (n=33)	100% (n=41)	15% (n=34)	38% (n=36)	44% (n=30)	20% (n=41)	4.9 days(n=40)
Significance[b]	No	No	Yes	No	No	No	Yes

[a] Antibiotic resistance was determined by antibiotic susceptibility testing with the disc diffusion method and refers to resistance to at least one of the antibiotics tested (tetracycline, naladixic acid, kanamycin, chloramphenicol, erythroymycin, ciprofloxacin), but always includes tetracycline resistance.

[b] Statistical significance ($p<0.05$) was calculated using a Fisher Exact Test.

(146; described in Chapter 2). As described above, pVir was found to be associated with pTet and an experiment was performed to determine if pVir could be conjugatively transferred with pTet to a recipient *C. jejuni* strain.

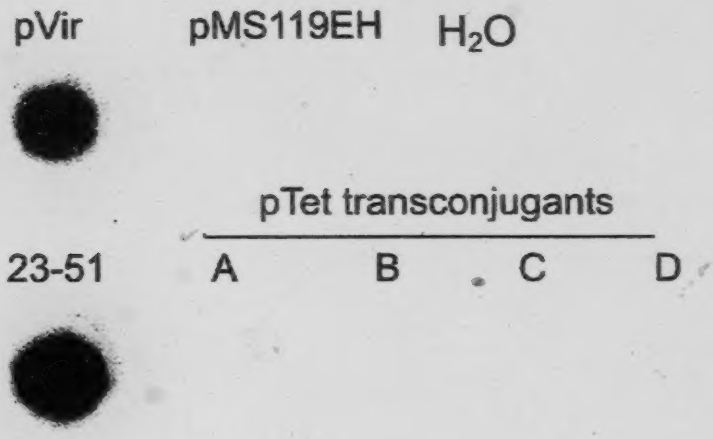
Mating of a clinical isolate (23-51) which contained both pVir and pTet was performed with a plasmid-free, tetracycline-susceptible, naladixic acid-resistant *C. jejuni* recipient strain (UA543). Transconjugants were selected with tetracycline and naladixic acid and plasmids were isolated from transconjugants and screened for pVir. Tetracycline-resistant transconjugants contained ~35-40 kb plasmids that carried *tet*(*O*) (pTet) but were negative for pVir in DNA-DNA hybridizations with the *cjp5* probe (Figure 3.4). This confirms that pVir was not transferred with pTet in conjugal mating between *C. jejuni* strains, which confirms the report of Bacon *et al.* (5).

3.4 Discussion

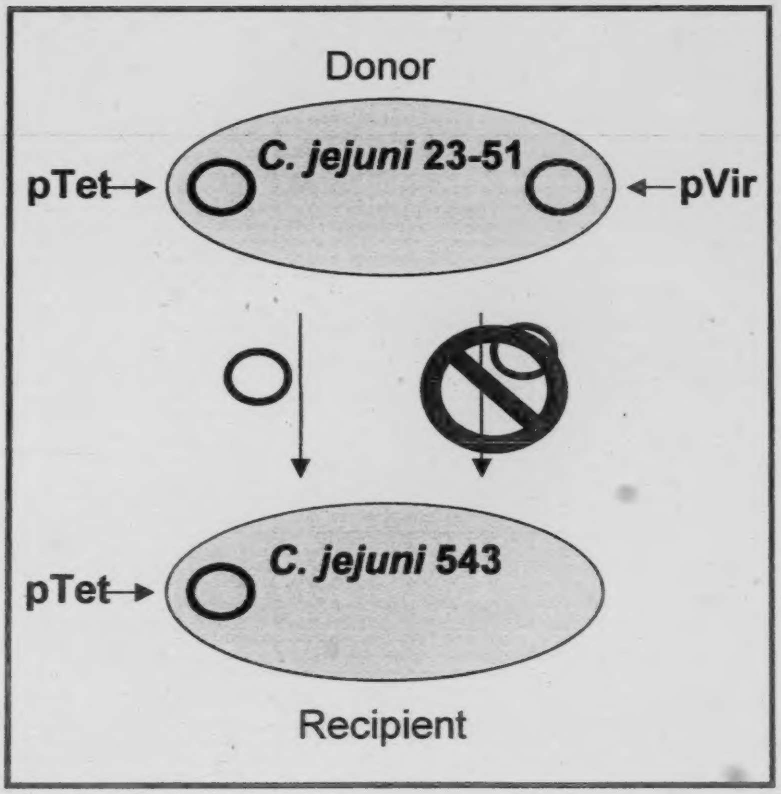
3.4.1 Clinical infections: Importance of pVir and antibiotic resistance

Although Bacon *et al.* (5, 6) have presented evidence for the role of pVir in the virulence of *C. jejuni*, their data is based upon *in vitro* cell culture experiments. The *in vivo* importance of pVir in *C. jejuni* human clinical infections has not been demonstrated to date. Only a small experiment in ferrets has shown any *in vivo* role in *C. jejuni* virulence (5). The present study has analyzed the clinical outcomes of *C. jejuni* infections and compared clinical

Figure 3.4. Screen for pVir in *C. jejuni* transconjugants, resulting from the mating of *C. jejuni* isolate 23-51 (containing both pVir and pTet) and *C. jejuni* UA543, a plasmid-free, tetracycline-susceptible and nalidixic acid-resistant recipient strain (as described in Materials and Methods). (A) pTet was isolated from all transconjugants screened, whereas pVir was not (A-D), indicating pVir was not transferred with pTet to other strains of *C. jejuni*. (B) Schematic diagram representing the transfer of pTet, but not pVir, to the recipient strain.


pVir
pMS119EH
H₂O
pTet transconjugants
23-51
A
B
C
D

A


Donor
pTet
C. jejuni 23-51
pVir
pTet
C. jejuni 543
Recipient

B

symptoms to the incidence of the pVir virulence plasmid and the occurrence of antibiotic resistance.

Clinical symptoms associated with *C. jejuni* infection were found to vary from cases with mild discomfort to severe cases with bloody diarrhea and various complications. Although the presence of the pVir virulence plasmid did not correlate with a higher frequency of most clinical symptoms, patients infected with a pVir-containing *C. jejuni* strain were more likely to have bloody stool than those infected with a strain lacking pVir. However, the association of pVir to the production of bloody stool was not a direct correlation, as patients infected with *C. jejuni* isolates lacking pVir also had bloody stool.

Infection with a number of enteric pathogens can result in the presence of blood in patient stool. For example, the EHEC Shiga-toxin can damage endothelial cells and lead to hemorrhagic colitis (62) or a massive inflammatory response in *Salmonella enterica* infections can damage the intestinal epithelium, leading to the leakage of blood and diarrhea (182). However, most cases of bloody diarrhea are due to *Campylobacter* infections, not *Salmonella* or *Shigella* infections (11, 119). The presence of blood in patient stool in *C. jejuni* gastroenteritis indicates the progression of the infection into the colon and rectum (133). Most patients with *Campylobacter* gastroenteritis have some element of colitis, which is characterized by a focal distribution of lesions in the intestinal mucosa (133). However, the mechanism by which invasive *C. jejuni* infections occur is currently unknown (162). It has been demonstrated *in vitro* that pVir is important for the invasion of an intestinal epithelial cell line (5, 6).

The association of bloody stool with the pVir virulence plasmid suggests that pVir may facilitate the invasion of the intestinal epithelium *in vivo*.

Infections with antibiotic-resistant strains of *C. jejuni* have been found to be associated with longer duration of clinical symptoms (94, 96). In one study, patients who did not take any form of diarrheal medication and were infected with a ciprofloxacin-resistant *C. jejuni* strain experienced a longer duration of diarrhea than those infected with ciprofloxacin-susceptible strains (96). Among patients treated with ciprofloxacin, prolonged diarrhea was also present in patients infected with ciprofloxacin-resistant *C. jejuni* (96). In this study, patients infected with antibiotic-resistant strains of *C. jejuni* had a longer duration of clinical symptoms than patients infected with antibiotic-susceptible strains.

In some clinical cases of *C. jejuni* infection, hospitalization may occur and is of some medical expense, at an estimated UK£779 (~CDN$1715) for managing care per patient (119). In this study, *C. jejuni* isolates from patients that were hospitalized (primarily for dehydration) did not vary significantly for pVir content or antibiotic susceptibility, accounting for an average of 18% of cases overall. In one case, the hospitalization of a child (age 22 months) was due to bacteremia, which can be a serious complication of *C. jejuni* infection (117). *C. jejuni* was isolated from a blood culture and yet pVir was not present in the fresh clinical isolate. In a study from Taiwan, high frequencies of resistance to macrolide and quinolone antibiotics were found in patients with *C. jejuni* bacteremia (90). The isolate responsible for the bacteremia case in this study was tetracycline-resistant, but was susceptible to all of the other antibiotics tested, including erythromycin, naladixic acid and ciprofloxacin.

3.4.2 Plasmids in *C. jejuni*: Co-incidence, transfer and stability

In their initial study on pVir, Bacon *et al*. (5) identified pVir in 10% (*n*=58) of fresh clinical isolates of *C. jejuni*. In the present study, the total sample of *C. jejuni* clinical isolates screened for pVir was nearly 2.5 times as large (*n*=137) and found the incidence of pVir to be higher (15%). Therefore, these two studies have reported a comparable level of pVir incidence. pTet was found in 74% of tetracycline-resistant isolates. In contrast, previous studies have found that all tetracycline-resistant *C. jejuni* isolates carry a plasmid (145).

This research has demonstrated that pVir virulence plasmids are found primarily in clinical isolates of *C. jejuni* that also contain a tetracycline-resistance plasmid. In contrast, approximately 85% of tetracycline-resistant *C. jejuni* isolates that contained pTet did not contain pVir (Table 3.5). Tetracycline resistance in *C. jejuni* is primarily mediated by plasmids which are transmissible to other strains of *C. jejuni*, but not to *E. coli* (145, 146). Bacon *et al*. (5) were not able to transfer a kanamycin-tagged pVir to other *Campylobacter* strains. In this study, pVir was not found to transfer with pTet to other strains of *C. jejuni*. The mechanism by which pVir is maintained within *C. jejuni* populations remains unknown. A potential explanation is that certain strains of *C. jejuni* possess a greater ability to acquire plasmids, perhaps through natural transformation.

The finding that pVir is present primarily in *C. jejuni* isolates that also contain pTet is of great interest. Variation in the invasion frequencies of *C. jejuni* isolates with pVir gene mutants have been observed (59) and may be due to the functional redundancy of T4SS genes found on pVir and pTet (6). This may, in part, explain the dependence of pVir on pTet. Furthermore, the association of pVir and pTet gives credence to the view that virulence and antibiotic resistance are linked (95), as both are characteristics commonly carried on mobile genetic elements. The integration of virulence and antibiotic resistance is discussed in Chapter 4, General Discussion.

Interestingly, virulence plasmids were readily isolated from frozen cultures and from cultures that underwent multiple laboratory passages without selection. Frozen isolates maintained both virulence and tetracycline resistance plasmids, regardless of their length of time frozen. Clearly, storing isolates of *C. jejuni* at -80°C does not appear to reduce the carriage of pVir, as frozen isolates contained pVir at higher levels than did fresh isolates. The stability of pVir has previously been observed, although spontaneous loss of pTet does occur (5, 6). pVir is stably maintained in the *C. jejuni* population and the focus on using fresh clinical isolates for analysis of virulence plasmids, as stressed by Bacon *et al.* (5), appears unnecessary.

From *C. jejuni* human clinical isolates studied from 1980-81, Taylor *et al.* (145) noted that some tetracycline-resistant strains contained a second plasmid of ~35 kb, which did not transfer along with the tetracycline resistance plasmids in mating experiments. It is possible that these “35 kb plasmids” were pVir, now known to be ~37.5 kb (6), as the results presented herein show that pVir is found primarily in isolates containing a tetracycline-resistance plasmid, but are not transferred along with pTet. The screening of these isolates for pVir with the *cjp5* DNA probe would be of great interest.

3.5 Conclusions and future directions

This study has considered the incidence of pVir and clinical symptoms in patients infected with *C. jejuni* and has found that the presence of pVir results in a higher frequency of bloody stool. What this study does not demonstrate is the invasiveness of the pVir containing *C. jejuni* clinical isolates. It is well known that strains of *C. jejuni* can differ in their level of invasion, with large variations in invasion frequency occurring among *C. jejuni* strains (61). Fearnely *et al.* (37) found that the invasion frequencies of *C. jejuni* strains are at either of two extremes: hyper-invasive or of very low invasion potential. Isolation of hyper-invasive strains from patients with bacteremia suggests that there may be a link between the invasive potential of a *C. jejuni* strain and the clinical outcome of the infection (37).

Currently, the only published studies on the *in vitro* role of pVir in *C. jejuni* invasion of cell cultures have used strain 81-176 (5, 6). However, recent developments have been made in the molecular typing of invasive *C. jejuni* isolates. Invasive and non-invasive *C. jejuni* isolates have been differentiated through random amplified polymorphic DNA (RAPD) analysis (17). This molecular typing technique uses PCR to amplify a potential invasion associated marker (IAM) from invasive isolates (17). Determining the incidence of the IAM among pVir containing *C. jejuni* clinical isolates would be of great interest for epidemiological screening, as the invasiveness of pVir containing clinical isolates may ultimately be correlated with the hyper-invasive phenotypes described by Fearnely *et al.* (37). However, the exact role that pVir plays in invasion is currently unknown.

What remains to be demonstrated is that *C. jejuni* clinical isolates containing pVir are the hyper-invasive phenotype described by some research groups (37) and that these same strains are associated with severe clinical symptoms. This possibility is further discussed in Chapter 4, General Discussion. Furthermore, the pVir T4SS is uncharacterized, as is the mechanism by which the secreted effector proteins act in invasion of intestinal epithelial cells. Resolution of these questions will lead to a greater understanding of the pathogenesis of *C. jejuni* and would be a substantial addition to the study of human enteric pathogens. Through this research on *C. jejuni*, strategies for controlling infections may be developed, such as the elimination of hyper-invasive strains through drugs targeting pVir. As the leading cause of bacterial gastroenteritis, *C. jejuni* is a major public health problem and limiting its spread and virulence would contribute to the control of food-borne bacterial infections.

Chapter 4

General Discussion

4 General Discussion

4.1 Virulence and antibiotic resistance: an integrated view?

The central concepts of this thesis deal with antibiotic resistance and virulence. This discussion will first consider the potential linkage of antibiotic resistance and virulence in *C. jejuni*, as well as in other bacterial pathogens. Both resistance and virulence are well-studied as individual areas of research, but rarely has an integrated view been considered. Nevertheless, Martinez and Baquero (95) considered the linkage of these topics extensively in their review. Antibiotic resistance and virulence are fundamental topics in medical microbiology, with especially important implications for enteric pathogens. The most important vehicles linking antibiotic resistance to virulence would be plasmids and other mobile genetic elements that are transmitted between bacteria by horizontal gene transfer (HGT) (111). Firstly, major enteric pathogens contain virulence plasmids that are essential for pathogenesis. Secondly, antibiotic resistance frequencies in these same pathogens have been increasing worldwide and are commonly transferred by plasmids or other mobile genetic elements. In this study, the link between resistance and virulence was demonstrated, as the incidence of the pVir virulence plasmid was found to be associated with the incidence of the pTet resistance plasmid in *C. jejuni* (see Chapter 3).

The integrated view of resistance and virulence can be explained with a hypothetical case study. Consider a patient infected with an enteric pathogen that has sought medical treatment, which will likely involve the administration of antibiotics. By definition, the pathogen is virulent, as it has been able to cause damage in the human host. To survive the antibiotic treatment, the pathogen must also be resistant to the antibiotic administered. As Martinez and Baquero (95) state, infective conditions and antibiotic treatment are two selective pressures that create a selective bottleneck from which only a small subset of the bacterial population survives to colonize the host. These two selective pressures are linked to mobile genetic elements and can be spread to the rest of the population through HGT (111).

The use and misuse of antibiotics has led to the emergence of drug-resistant enteric pathogens and may potentially select for virulence determinants, as discussed above. Together, the selection of antibiotic resistance and virulence traits produce bacterial populations that pose significant public health threats. Although this has a solid theoretical basis, there is only limited evidence *in vivo* for antibiotic resistant strains being more virulent. One reason for this observation is that antibiotic resistance is believed to have a negative effect on bacterial fitness (3). Counteracting this negative effect is the advantage of possessing virulence determinants, as the pathogenic process would allow the pathogen to access host habitats that are normally restrictive (95). Also, it could be argued that antibiotic resistance is a virulence factor that contributes to the colonization of the host, as resistance is similar to other systems that evade host defenses (95).

In vivo data for *C. jejuni* suggests that antibiotic-resistant strains are more virulent, causing prolonged and more severe clinical symptoms (156). Patients infected with ciprofloxacin-resistant *C. jejuni* strains have been found to undergo increased hospitalization rates and a longer duration of clinical symptoms (94). Similar effects have been observed for other enteric pathogens, such as *Salmonella* sp. infections which lead to increased hospitalization rates (82). The effect is not due to ineffective antibiotic treatment, as the studies controlled for antibiotic resistant and susceptible strains in both treated and untreated cases. This study has demonstrated antibiotic-resistant strains of *C. jejuni* result in infections that produce longer duration of clinical symptoms (see Chapter 3). Medical researchers must now view the potential for continued increases in antibiotic resistance among enteric pathogens with the impact on virulence.

4.2 Invasion as a common strategy in enteric pathogens

The virulence of enteric pathogens is multi-factoral, as bacteria have evolved various mechanisms to induce damage in the host. Although different pathogenic strategies are used by enteric pathogens to undermine host cellular functions, the common outcome is internalization or invasion of the intestinal epithelium (41). *Salmonella* and *Shigella* spp. are both invasive pathogens that use a T3SS to induce their uptake by exploiting host cells to rearrange their cytoskeletons (40). Furthermore, they have common cellular targets (such as M-cells) and shared strategies for intracellular survival (41).

Invasion is also a main component *C. jejuni* pathogenesis (61). Unlike *Salmonella* or *Shigella*, the *C. jejuni* genome does not appear to contain a T3SS (113). However, as discussed in Chapter 1 and 3, the pVir virulence plasmid encodes 7 homologues of T4SS proteins (6), which resemble the T4SS in a closely related bacterium, *H. pylori*. The importance of T4SSs to virulence is well established, as it is responsible for the secretion of effector proteins in a number of bacterial pathogens (20). Evidence from invasion assays suggests pVir is necessary for an invasive phenotype in *C. jejuni* 81-176 (5). However, the transfer of pVir into the non-invasive *C. jejuni* strain, NCTC 11168, does not confer the ability to invade cells *in vitro*, suggesting other factors are also required for full invasion. It is likely that the pVir T4SS is responsible for secreting an effector protein that is important in invasion. Bacon *et al.* (6) have suggested that Cjp32 may be the effector protein, but recent evidence shows that Cjp29 is the likely effector (59). Thus, the virulence of a particular *C. jejuni* strain may be dependent on the pVir virulence plasmid, which through its T4SS could confer a hyper-invasive phenotype and allows for the invasion of the intestinal mucosa.

A growing concept in *C. jejuni* research is that sub-populations of *C. jejuni* may exist that differ in their pathogenesis (61, 75). That is, certain subsets of the *C. jejuni* population may use different mechanisms to produce disease. These different pathogenic mechanisms could potentially include one group of adherent, but non-invasive strains that produce toxins and a second group of hyper-invasive strains that efficiently penetrate the intestinal epithelial barrier

(75). In the former, diarrhea may be a common symptom, while bloody stool and diarrhea may be the predominant clinical feature in the latter. This study has identified an association between the incidence of the pVir virulence plasmid and a higher occurrence of bloody stool, a symptom indicative of an invasive infection. Whether or not the pVir virulence plasmid is the defining feature of a hyper-invasive subset of *C. jejuni* strains remains to be determined.

There is widespread genetic diversity among the *Campylobacter* genus and within *Campylobacter* species. Currently, studies on the genetic structure of *C. jejuni* populations are inconclusive (100) and it is unknown whether there are distinct clonal lineages of pathogenic types. Evidence suggests that there is variation in the virulence of *C. jejuni* cells, exemplified by different invasion frequencies among clinical strains (61). If sub-populations of *C. jejuni* that have different mechanisms of pathogenesis, this would be analogous to the various serotypes of pathogenic *E. coli*: EHEC, EPEC, EIEC and EAEC. It is possible that a common theme among enteric pathogens is that sub-populations of the same species use a variety of mechanisms to produce disease in the host. The clinical outcomes of these enteric infections are dependent on the virulence of the infecting pathogen and the host response. In *C. jejuni* infections, the pathogen has been classified as the primary contributor to host damage, with strong immune responses contributing to prolonged or rare complicated illnesses (18). Given the complex nature of clinical infections and the role of the host response, the clinical outcome of infection with a *C. jejuni* strain may stem from both the pVir virulence plasmid and other host factors.

4.3 At the Crossroads: Antibiotics, agriculture and medical treatment

This study has identified increased rates of tetracycline resistance in *C. jejuni* and studies from around the globe show similar increases in resistance to other antibiotics, such as ciprofloxacin. The reasons for increasing antibiotic-resistance frequencies in *C. jejuni* remain unknown, but the use of antibiotics in the agriculture industry may be a contributing factor. The use of antibiotics in both humans (for treatment) and in animals (for treatment and growth promotion) selects for antibiotic-resistant bacterial populations (173). By using antibiotics in animal agriculture at sub-therapeutic levels, resistant bacteria are selected for and can be transmitted to humans through contaminated food. This is a serious medical problem, as treatment of major zoonotic pathogens such as *Salmonella* sp., *E. coli* and *Campylobacter* may be compromised.

The ability of antibiotics to promote growth in animals was first discovered in the 1940's and has been used ever since in animal agriculture. By feeding antibiotics to livestock at sub-therapeutic doses, animals have shown increased growth rates and dairy cows have higher milk yields (32). Animals are able to gain up to 5% more body weight when fed antibiotics sub-therapeutically (177). The amount of antibiotics used in animal agriculture is striking: for example, in Denmark 24 kg of the glycopeptide vancomycin is used annually for human therapy while 24,000 kg of the glycopeptide avoparcin are used in animal feed (177).

Tetracyclines are used for treating infections, prophylaxis and as growth promoters in animal agriculture (32, 98). For example, in the United States, tetracycline is used in the poultry industry to treat infections in broiler chicken flocks and chlorotetracycline is used as a growth promoter (98). This does not appear to be the case in Canada. There is an apparent connection to tetracycline-resistance frequencies in commercially available poultry products, as in a recent survey of retail meats, 82% of *Campylobacter* isolates were tetracycline-resistant (46). A major source of human *C. jejuni* infections is through contaminated poultry products (10, 26). T[illegible]s, poultry treated with tetracyclines may be the main route for the acquisition of tetracycline resistance in *C. jejuni* clinical isolates. Determining the rate of tetracycline resistance among poultry isolates in the Alberta agriculture industry would provide important evidence for this potential link.

There is strong evidence that use of fluoroquinolones in agriculture for growth promotion and treatment of animal infections has led to the observed increase in fluoroquinolone-resistant *C. jejuni* infections in humans (33). In Alberta, the Department of Agriculture reports that fluoroquinolone (enrofloxacin) resistance frequencies among animal isolates of *C. jejuni* are approximately 12% (M. VanderKop, personal communication). Thus, as the ciprofloxacin resistance rate in human clinical isolates was only at 1.5%, there does not appear to be a relationship between fluoroquinolone resistance in human clinical isolates and animal isolates in Alberta, Canada. These results are in contrast to several studies that suggest fluoroquinolone resistance in animal isolates of C. *jejuni* leads to higher resistance in human clinical isolates (33, 134).

The emergence of fluoroquinolone and macrolide resistance in C. *jejuni* isolated from animal agricultural sources is a serious medical concern. The potential for transmission of isolates resistant to these front-line antibiotics up the food chain may compromise the treatment of human C. *jejuni* infections (34). This is especially a concern for treatment of C. *jejuni* enteritis in AIDS patients, in which there is documented evidence of the acquisition of resistance to multiple antibiotics during treatment (152).

In this study, multiple antibiotic resistance in C. *jejuni* was found to always include resistance to tetracycline. Chopra and Roberts (19) note that the use of tetracycline quickly selects for tetracycline resistance among Gram-negative bacteria and that tetracycline genes are commonly carried on the same genetic elements as other antibiotic resistance genes. Thus, selection for one antibiotic will result in selection for all resistance determinants carried on the element. A similar problem exists for antibiotic resistance gene clusters carried in integrons.

The Swann Committee report of 1969 (UK) was first to address the concern over antibiotic use in animal agriculture and increasing antibiotic resistance in bacteria (32). Since, the World Health Organization has recommended the elimination of all anti-microbial growth promoters that are used in human therapy (175). In the US, antibiotic use in animal agriculture has been addressed by the Food and Drug Administration, which in 2000 proposed that fluoroquinolones not be used in poultry. This decision was based on evidence from published studies, surveillance programs and risk assessments, all which showed that fluoroquinolone use in the poultry industry has led to the emergence of fluoroquinolone resistant *C. jejuni* infections in humans (173). The problem with antibiotic use in animals and resistant infections in humans extends beyond *C. jejuni*, as recent studies have shown a similar problem with *E. coli* (161). As White *et al*. (173) state, the use and misuse of antibiotics has produced an unparalleled level of selection in the history of bacterial evolution. This is a serious public health challenge that must be dealt with, as it is possible that bacterial pathogens may emerge that are resistant to all available antibiotics.

4.4 Conclusion

It was only three decades ago that the threat of infectious disease was thought by many in the medical profession to have been conquered (42). Through the acquisition of antibiotic resistance and virulence genes on plasmids and other mobile genetic elements, enteric bacterial pathogens have maintained their status as a serious threat to public health worldwide. As Witte (177) wrote, "evolving bacterial populations do not respect geographical boundaries."

Although the development of a vaccine to prevent *C. jejuni* infection has been proposed, the true answer for control of transmission may be much simpler. Public education programs that inform the population on proper food handling techniques and strict adherence to regulations by the food industry are critical to the prevention of *C. jejuni* transmission. Such education programs and regulations are necessary to prevent food-borne diseases, as newly identified food-borne zoonotic pathogens are being discovered at a rapid rate (141). With the emergence of new diseases and the re-emergence of old pathogens, food-borne diseases will continue to challenge the medical system. The spread of antibiotic resistance and virulence genes among these pathogens further complicates effective treatment. While continued research and surveillance will be important in resolving this problem, the use of common sense among individuals handling food products is an excellent starting point to prevent food-borne infections.

5 References

1. **Aarestrup, F. M., E. M. Nielsen, M. Madsen, and J. Engberg.** 1997. Antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. from humans, pigs, cattle, and broilers in Denmark. Antimicrob Agents Chemother **41**:2244-2250.
2. **Ambrosio, R. E., and A. J. Lastovica.** 1983. Rapid screening procedure for detection of plasmids *Campylobacter*. S Afr J Sci **79:**110-111.
3. **Andersson, D. I., and B. R. Levin.** 1999. The biological cost of antibiotic resistance. Curr Opin Microbiol **2**.
4. **Austen, R. A., and T. J. Trust.** 1980. Detection of plasmids in the related group of the genus *Campylobacter*. FEMS Microbiol Lett **8:**201-204.
5. **Bacon, D. J., R. A. Alm, D. H. Burr, L. Hu, D. J. Kopecko, C. P. Ewing, T. J. Trust, and P. Guerry.** 2000. Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. Infect Immun **68:**4384-4390.
6. **Bacon, D. J., R. A. Alm, L. Hu, T. E. Hickey, C. P. Ewing, R. A. Batchelor, T. J. Trust, and P. Guerry.** 2002. DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81-176. Infect Immun **70:**6242-6250.
7. **Bacon, D. J., C. M. Szymanski, D. H. Burr, R. P. Silver, R. A. Alm, and P. Guerry.** 2001. A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. Mol Microbiol **40:**769-777.
8. **Berndtson, E., M. Tivemo, and A. Engvall.** 1992. Distribution and numbers of *Campylobacter* in newly slaughtered broiler chickens and hens. Int J Food Microbiol **15:**45-50.
9. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis **157:**472-479.
10. **Blaser, M. J.** 1997. Epidemiologic and clinical features of *Campylobacter jejuni* infections. J Infect Dis **176 Suppl 2:**S103-S105.
11. **Blaser, M. J., J. G. Wells, R. A. Feldman, R. A. Pollard, and J. R. Allen.** 1983. *Campylobacter enteritis* in the United States. A multicenter study. Ann Intern Med **98:**360-365.
12. **Bradbury, W. C., M. A. Marko, J. N. Hennessy, and J. L. Penner.** 1983. Occurrence of plasmid DNA in serologically defined strains of *Campylobacter jejuni* and *Campylobacter coli*. Infect Immun **40:**460-463.
13. **Bradbury, W. C., and D. L. Munroe.** 1985. Occurrence of plasmids and antibiotic resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from healthy and diarrheic animals. J Clin Microbiol **22:**339-346.

14. **Broman, T., H. Palmgren, S. Bergstrom, M. Sellin, J. Waldenstrom, M. L. Danielsson-Tham, and B. Olsen.** 2002. *Campylobacter jejuni* in black-headed gulls (*Larus ridibundus*): prevalence, genotypes, and influence on *C. jejuni* epidemiology. J Clin Microbiol **40:**4594-4602.
15. **Burdett, V., J. Inamine, and S. Rajagopalan.** 1982. Heterogeneity of tetracycline resistance determinants in *Streptococcus* spp. J Bacteriol **149:**995-1004.
16. **Buzby, J. C., T. Roberts, C. T. Jordan Lin, and J. M. MacDonald.** 1996. Bacterial foodborne diseases: Medical costs and productivity losses Agricultural Economics Report No. 741. Economic Research Service, United States Department of Agriculture.
17. **Carvalho, A. C., G. M. Ruiz-Palacios, P. Ramos-Cervantes, L. E. Cervantes, X. Jiang, and L. K. Pickering.** 2001. Molecular characterization of invasive and noninvasive *Campylobacter jejuni* and *Campylobacter coli* isolates. J Clin Microbiol **39:**1353-1359.
18. **Casadevall, A., and L. Pirofski.** 1999. Host-pathogen interactions: Redefining the basic concepts of virulence and pathogenicity. Infect Immun **67:**3703-3713.
19. **Chopra, I., and M. Roberts.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev **65:**232-260.
20. **Christie, P. J.** 2001. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. Mol Microbiol **40:**294-305.
21. **Chuma, T., T. Ikeda, T. Maeda, N. Hidekazu, and K. Okamoto.** 2001. Antimicrobial susceptibilities of *Campylobacter* strains isolated from broilers in the southern part of Japan from 1995 to 1999. J Vet Med Sci **63:**1027-1029.
22. **Connell, S. R.** 2003. Interaction of the tetracycline resistance protein Tet(O) with the bacterial ribosome. University of Alberta, Edmonton, AB.
23. **Connell, S. R., C. A. Trieber, G. P. Dinos, E. Einfeldt, D. E. Taylor, and K. H. Nierhaus.** 2003. Mechanism of Tet(O)-mediated tetracycline resistance. EMBO J **22:**945-953.
24. **Connell, S. R., C. A. Trieber, U. Stelzl, E. Einfeldt, D. E. Taylor, and K. H. Nierhaus.** 2002. The tetracycline resistance protein Tet(O) perturbs the conformation of the ribosomal decoding centre. Mol Microbiol **45:**1463-1472.
25. **Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier.** 1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol Mol Biol Rev **62:**1315-1352.
26. **Corry, J. E., and H. I. Atabay.** 2001. Poultry as a source of *Campylobacter* and related organisms. Symp Ser Soc Appl Microbiol:96S-114S.
27. **Crushell, E., S. Harty, F. Sharif, and B. Bourke.** 2003. Enteric *Campylobacter*: Purging its secrets. Ped Rev **Submitted**.

28. **Davies, J.** 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science **264:**375-382.
29. **Davies, J. E.** 1997. Origins, acquisition and dissemination of antibiotic resistance determinants. Ciba Found Symp **207:**15-27.
30. **De Ungria, M. C., D. Tillett, B. A. Neilan, P. T. Cox, and A. Lee.** 1998. A novel method of extracting plasmid DNA from *Helicobacter* species. Helicobacter **3:**269-277.
31. **Drlica, K.** 1999. Mechanism of fluoroquinolone action. Curr Opin Microbiol **2:**504-508.
32. **Edqvist, L., and K. B. Pedersen.** 2001. Antimicrobials as growth promoters: resistance to common sense, p. 93-100. *In* P. Harremoes, D. Gee, M. MacGarvin, A. Stirling, and J. Keys (ed.), The Precautionary Principle in the 20th Century. European Environmental Protection Agency.
33. **Endtz, H. P., G. J. Ruijs, B. van Klingeren, W. H. Jansen, T. van der Reyden, and R. P. Mouton.** 1991. Quinolone resistance in campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother **27:**199-208.
34. **Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin.** 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. Emerg Infect Dis **7:**24-34.
35. **Falkow, S.** 1996. The evolution of pathogenicity in *Escherichia*, *Shigella*, and *Salmonella*. *In* F. C. Nierdhart (ed.), *Escherichia coli* and *Salmonella typhimirium*, 2nd ed, vol. 2. ASM Press, Washington, D.C.
36. **Fauchere, J. L., A. Rosenau, M. Veron, N. Moyen, S. Richard, and A. Pfister.** 1986. Association with HeLa cells of *Campylobacter jejuni* and *Campylobacter coli* isolated from human feces. Infect Immun **54:**283-287.
37. **Fearnley, C., G. Manning, J. Frost, C. W. Penn, and D. G. Newell.** 2001. The invasiveness of *C. jejuni* isolates in relation to the source and host presentation of disease. Int J Med Micro **291:**124.
38. **Fernandez, H., M. Mansilla, and V. Gonzalez.** 2000. Antimicrobial susceptibility of *Campylobacter jejuni* subsp. *jejuni* assessed by E-test and double dilution agar method in Southern Chile. Mem Inst Oswaldo Cruz **95:**247-249.
39. **Finlay, B. B., and S. Falkow.** 1990. Salmonella interactions with polarized human intestinal Caco-2 epithelial cells. J Infect Dis **162:**1096-1106.
40. **Galan, J. E., and A. Collmer.** 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284:**1322-1328.

41. **Galan, J. E., and P. J. Sansonetti.** 1996. Molecular and cellular bases of *Salmonella* and *Shigella* interactions with host cells. *In* F. C. Nierdhart (ed.), *Escherichia coli* and *Salmonella typhimirium*, vol. 2. ASM Press, Washington, D.C.

42. **Garrett, L.** 1994. The Coming Plague: Newly Emerging Diseases in a World Out of Balance. Penguin Books, New York, New York.

43. **Gaudreau, C., and H. Gilbert.** 1998. Antimicrobial resistance of clinical strains of *Campylobacter jejuni* subsp. *jejuni* isolated from 1985 to 1997 in Quebec, Canada. Antimicrob Agents Chemother **42:**2106-2108.

44. **Gaudreau, C., and H. Gilbert.** 1997. Comparison of disc diffusion and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* subsp. *jejuni* and *Campylobacter coli*. J Antimicrob Chemother **39:**707-712.

45. **Gaunt, P. N., and L. J. Piddock.** 1996. Ciprofloxacin resistant *Campylobacter* spp. in humans: an epidemiological and laboratory study. J Antimicrob Chemother **37:**747-757.

46. **Ge, B., S. Bodeis, R. D. Walker, D. G. White, S. Zhao, P. F. McDermott, and J. Meng.** 2002. Comparison of the Etest and agar dilution for in vitro antimicrobial susceptibility testing of *Campylobacter*. J Antimicrob Chemother **50:**487-494.

47. **Goosney, D. L., D. G. Knoechel, and B. B. Finlay.** 1999. Enteropathogenic *E. coli*, *Salmonella*, and *Shigella*: masters of host cell cytoskeletal exploitation. Emerg Infect Dis **5:**216-223.

48. **Grant, C. C., M. E. Konkel, W. Cieplak, Jr., and L. S. Tompkins.** 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect Immun **61:**1764-1771.

49. **Guerry, P., C. M. Szymanski, M. M. Prendergast, T. E. Hickey, C. P. Ewing, D. L. Pattarini, and A. P. Moran.** 2002. Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness *in vitro*. Infect Immun **70:**787-793.

50. **Guiney, D. G., F. C. Fang, M. Krause, and S. Libby.** 1994. Plasmid mediated virulence genes in non-typhoidal *Salmonella* serovars. FEMS Microbiol Lett **124:**1-9.

51. **Guiney, D. G., F. C. Fang, M. Krause, S. Libby, N. A. Buchmeier, and J. Fierer.** 1995. Biology and clinical significance of virulence plasmids in *Salmonella* serovars. Clin Infect Dis **21 Supp 2:**S146-S151.

52. **Hadden, R. D., and N. A. Gregson.** 2001. Guillain--Barre syndrome and *Campylobacter jejuni* infection. Symp Ser Soc Appl Microbiol:145S-154S.

53. **Hald, B., A. Wedderkopp, and M. Madsen.** 2000. Thermophilic *Campylobacter* spp. in Danish broiler production: a cross sectional survey and a retrospective analysis of risk factors for the occurrence in broiler flocks. Avian Path **29:**123-131.

54. **Harnett, N., S. McLeod, Y. A. Yong, C. Hewitt, M. Vearncombe, and C. Krishnan.** 1995. Quinolone resistance in clinical strains of *Campylobacter jejuni* and *Campylobacter coli*. J Antimicrob Chemother **36**:269-270.
55. **Harris, L. A., S. M. Logan, P. Guerry, and T. J. Trust.** 1987. Antigenic variation of *Campylobacter* flagella. J Bacteriol **169**.
56. **Health Canada, H. P. B.** 2003, posting date. Notifiable Diseases On-Line. Health Protection Branch, Health Canada. [Online.]
57. **Hickey, T. E., A. L. McVeigh, D. A. Scott, R. E. Michielutti, A. Bixby, S. A. Carroll, A. L. Bourgeois, and P. Guerry.** 2000. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun **68**:6535-6541.
58. **Hoge, C. W., J. M. Gambel, C. Srijan, C. Pitarangsi, and P. Echeverria.** 1998. Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clin Infect Dis **26**:341-345.
59. **Hu, L., A. Kiavand, C. P. Ewing, D. J. Bacon, and P. Guerry.** 2001. Characterization of a plasmid-encoded type IV secretion system in *Campylobacter jejuni* 81-176. Int J Med Micro **291**:120.
60. **Hu, L., and D. J. Kopecko.** 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. Infect Immun **67**:4171-4182.
61. **Hu, L., and D. J. Kopecko.** 2000. Interactions of *Campylobacter* with eukaryotic cells: Gut luminal colonization and mucosal invasion mechanisms. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.
62. **Jacewicz, M. S., D. W. K. Acheson, D. G. Binion, G. A. West, L. L. Lincicome, C. Fiocchi, and G. T. Keusch.** 1999. Responses of human intestinal microvascular endothelial cells to Shiga toxins 1 and 2 and pathogenesis of hemorrhagic colitis. Infect Immun **67**:1439-1444.
63. **Jensen, L. B., and F. M. Aarestrup.** 2001. Macrolide resistance in *Campylobacter coli* of animal origin in Denmark. Antimicrob Agents Chemother **45**:371-372.
64. **Johnson, W. M., and H. Lior.** 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. Microb Pathog **4**:115-126.
65. **Jones, K.** 2001. Campylobacters in water, sewage and the environment. Symp Ser Soc Appl Microbiol:68S-79S.
66. **Kaper, J. B.** 1998. Enterohemorrhagic *Escherichia coli*. Curr Opin Microbiol **1**:103-108.
67. **Karmali, M. A., S. De Grandis, and P. C. Fleming.** 1981. Antimicrobial susceptibility of *Campylobacter jejuni* with special reference to resistance patterns of Canadian isolates. Antimicrob Agents Chemother **19**:593-597.

68. **Karmali, M. A., J. L. Penner, F. C. Fleming, A. Williams, and J. N. Hennessy.** 1983. The serotype and biotype distribution of clinical isolates of *Campylobacter jejuni* and *Campylobacter coli* over a three-year period. J Infect Dis **147**:243-246.

69. **Kazwala, R. R., J. D. Collins, J. Hannan, R. A. P. Crinion, and H. O'Mahony.** 1990. Factors responsible for the introduction and spread of *Campylobacter jejuni* infection in commerical poultry production. Vet Rec **126**:305-306.

70. **Konkel, M. E., S. G. Garvis, S. L. Tipton, D. E. Anderson, Jr., and W. Cieplak, Jr.** 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol Microbiol **24**:953-963.

71. **Konkel, M. E., and L. A. Joens.** 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect Immun **57**:2984-2990.

72. **Konkel, M. E., L. A. Joens, and P. F. Mixter.** 2000. Molecular characterization of *Campylobacter jejuni* virulence determinants. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.

73. **Konkel, M. E., B. J. Kim, V. Rivera-Amill, and S. G. Garvis.** 1999. Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. Mol Microbiol **32**:691-701.

74. **Konkel, M. E., D. J. Mead, S. F. Hayes, and W. Cieplak, Jr.** 1992. Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. J Infect Dis **166**:308-315.

75. **Kopecko, D. J., L. Hu, and K. J. Zaal.** 2001. *Campylobacter jejuni*--microtubule-dependent invasion. Trends Microbiol **9**:389-396.

76. **Korlath, J. A., M. T. Osterholm, L. A. Judy, J. C. Forfang, and R. A. Robinson.** 1985. A point-source outbreak of campylobacteriosis associated with consumption of raw milk. J Infect Dis **152**:592-596.

77. **Lammerding, A. M., M. M. Garcia, E. D. Mann, Y. Robinson, W. J. Dorward, R. B. Trusscott, and F. Tittiger.** 1988. Prevalence of salmonella and thermophilic *Campylobacter* in fresh pork, beef, veal and poultry in Canada. J Food Prot **51**.

78. **Lariviere, L. A., C. L. Gaudreau, and F. F. Turgeon.** 1986. Susceptibility of clinical isolates of *Campylobacter* jejuni to twenty-five antimicrobial agents. J Antimicrob Chemother **18**:681-685.

79. **Lastovica, A. J., and M. B. Skirrow.** 2000. Clinical significance of *Campylobacter* and related species other than *Campylobacter jejuni* and *C. coli*. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.

80. **Lawley, T. D., M. W. Gilmour, J. E. Gunton, D. M. Tracz, and D. E. Taylor.** 2003. Functional and Mutational Analysis of Conjugative Transfer Region 2 (Tra2) from the IncHI1 Plasmid R27. J Bacteriol **185**:581-591.

81. **Lee, C. Y., C. L. Tai, S. C. Lin, and Y. T. Chen.** 1994. Occurrence of plasmids and tetracycline resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from whole market chickens and clinical samples. Int J Food Microbiol **24:**161-170.

82. **Lee, L. A., N. D. Puhr, E. K. Maloney, N. H. Bean, and R. V. Tauxe.** 1994. Increase in antimicrobial-resistant *Salmonella* infections in the United States, 1989-1990. J Infect Dis **170:**128-134.

83. **Leonard, E. E., T. Takata, M. J. Blaser, S. Falkow, L. Tompkins, and E. C. Gaynor.** 2003. Use of an open-reading frame specific *Campylobacter jejuni* DNA microarray as a new genotyping tool for studying epidemiologically related isolates. J Infect Dis **187:**691-694.

84. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J Infect Dis **155:**377-389.

85. **Levy, S. B.** 1978. Emergence of antibiotic-resistant bacteria in the intestinal flora of farm inhabitants. J Infect Dis **137:**689-690.

86. **Levy, S. B., G. B. FitzGerald, and A. B. Macone.** 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. N Engl J Med **295:**583-588.

87. **Li, C. C., C. H. Chiu, J. L. Wu, Y. C. Huang, and T. Y. Lin.** 1998. Antimicrobial susceptibilities of *Campylobacter jejuni* and coli by using E-test in Taiwan. Scand J Infect Dis **30:**39-42.

88. **Lin, J., L. O. Michel, and Q. Zhang.** 2002. CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. Antimicrob Agents Chemother **46:**2124-2131.

89. **Lind, L., E. Sjogren, C. Welinder-Olsson, and B. Kaijser.** 1989. Plasmids and serogroups in *Campylobacter jejuni*. Apmis **97:**1097-1102.

90. **Lu, P. L., P. R. Hsueh, C. C. Hung, S. C. Chang, K. T. Luh, and C. Y. Lee.** 2000. Bacteremia due to *Campylobacter* species: high rate of resistance to macrolide and quinolone antibiotics. J Formos Med Assoc **99:**612-617.

91. **Lucey, B., B. Cryan, F. O'Halloran, P. G. Wall, T. Buckley, and S. Fanning.** 2002. Trends in antimicrobial susceptibility among isolates of *Campylobacter* species in Ireland and the emergence of resistance to ciprofloxacin. Vet Rec **151:**317-20.

92. **Luo, N., and Q. Zhang.** 2001. Molecular characterization of a cryptic plasmid from *Campylobacter jejuni*. Plasmid **45:**127-133.

93. **Manavathu, E. K., K. Hiratsuka, and D. E. Taylor.** 1988. Nucleotide sequence analysis and expression of a tetracycline-resistance gene from *Campylobacter jejuni*. Gene **62:**17-26.

94. **Marano, N., D. Vugia, T. Fiorentino, S. Segler, M. Carter, H. Kassenborg, K. Smith, S. Zansky, K. Hollinger, F. Angulo, and E. F. W. Group.** 2000. Presented at the International Conference on Emerging Infectious Diseases, Atlanta, GA, July, 2000.

95. **Martinez, J. L., and F. Baquero.** 2002. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. Clin Microbiol Rev **15:**647-679.

96. **McClellan, J., S. Rossiter, K. Joyce, K. Starney, A. Anderson, and N. w. group.** 2002. Presented at the International Conference on Emerging Infectious Diseases, Atlanta, GA, March, 2002.

97. **McDermott, P. F., S. M. Bodeis, L. L. English, D. G. White, R. D. Walker, S. Zhao, S. Simjee, and D. D. Wagner.** 2002. Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. J Infect Dis **185:**837-840.

98. **McEwen, S. A., and P. J. Fedorka-Cray.** 2002. Antimicrobial use and resistance in animals. Clin Infect Dis **34 Suppl 3:**S93-S106.

99. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg Infect Dis **5:**607-625.

100. **Meinersmann, R. J., C. M. Patton, G. M. Evins, I. K. Wachsmuth, and P. I. Fields.** 2002. Genetic diversity and relationships of *Campylobacter* species and subspecies. Int J Syst Evol Microbiol **52:**1789-1797.

101. **Melby, K., O. P. Dahl, L. Crisp, and J. L. Penner.** 1990. Clinical and serological manifestations in patients during a waterborne epidemic due to *Campylobacter jejuni*. J Infect **21:**309-316.

102. **Mohran, Z. S., R. R. Arthur, B. A. Oyofo, L. F. Peruski, M. O. Wasfy, T. F. Ismail, and J. R. Murphy.** 1998. Differentiation of *Campylobacter* isolates on the basis of sensitivity to boiling in water as measured by PCR-detectable DNA. Appl Environ Microbiol **64:**363-365.

103. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect Immun **70:**6665-6671.

104. **Moore, J. E., M. Crowe, N. Heaney, and E. Crothers.** 2001. Antibiotic resistance in *Campylobacter* spp. isolated from human faeces (1980-2000) and foods (1997-2000) in Northern Ireland: an update. J Antimicrob Chemother **48:**455-457.

105. **Nachamkin, I.** 1994. Antimicrobial susceptibilities of *Campylobacter jejuni* and *Campylobacter coli* to ciprofloxacin, erythromycin and tetracycline from 1982 to 1992. Med. Microbiol. Lett. **3:**300-305.

106. **Nachamkin, I., B. M. Allos, and T. Ho.** 1998. *Campylobacter* species and Guillian-Bare syndrome. *Clin Microbiol Rev* **11:**555-567.

107. **Nachamkin, I., J. Engberg, and F. M. Aarestrup.** 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* spp. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.

108. **Nachamkin, I., H. Ung, and M. Li.** 2002. Increasing fluoroquinolone resistance in *Campylobacter jejuni*, Pennsylvania, USA, 1982-2001. Emerg Infect Dis **8:**1501-1503.

109. **Nakajima, Y.** 1999. Mechanisms of bacterial resistance to macrolide antibiotics. J Infect Chemother **5:**61-74.

110. **Navarro-Garcia, F., A. Canizalez-Roman, J. Luna, C. Sears, and J. P. Nataro.** 2001. Plasmid-encoded toxin of enteroaggregative *Escherichia coli* is internalized by epithelial cells. Infect Immun **69:**1053-1060.

111. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299-304.

112. **Pallen, M., B. W. Wren, and J. Parkhill.** 1999. 'Going wrong with confidence': misleading sequence analyses of CiaB and *clpX*. Mol Microbiol **34:**195.

113. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665-668.

114. **Payot, S., A. Cloeckaert, and E. Chaslus-Dancla.** 2002. Selection and characterization of fluoroquinolone-resistant mutants of *Campylobacter jejuni* using enrofloxacin. Microb Drug Resist **8:**335-343.

115. **Pei, Z., C. Burucoa, B. Grignon, S. Baqar, X. Z. Huang, D. J. Kopecko, A. L. Bourgeois, J. L. Fauchere, and M. J. Blaser.** 1998. Mutation in the *peb1A* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect Immun **66:**938-943.

116. **Penner, J. L.** 1988. The genus *Campylobacter*: a decade of progress. Clin Microbiol Rev **1:**157-172.

117. **Pigrau, C., R. Bartolome, B. Almirante, A. M. Planes, J. Gavalda, and A. Pahissa.** 1997. Bacteremia due to *Campylobacter* species: clinical findings and antimicrobial susceptibility patterns. Clin Infect Dis **25:**1414-1420.

118. **Prasad, K. N., S. K. Mathur, T. N. Dhole, and A. Ayyagari.** 1994. Antimicrobial susceptibility and plasmid analysis of *Campylobacter jejuni* isolated from diarrhoeal patients and healthy chickens in northern India. J Diarrhoeal Dis Res **12:**270-273.

119. **Rao, G. G., and M. Fuller.** 1992. A review of hospitalized patients with bacterial gastroenteritis. J Hosp Infect **20:**105-111.

120. **Reina, J., M. J. Ros, and A. Serra.** 1994. Susceptibilities to 10 antimicrobial agents of 1,220 *Campylobacter* strains isolated from 1987 to 1993 from feces of pediatric patients. Antimicrob Agents Chemother **38:**2917-2920.

121. **Rivera-Amill, V., B. J. Kim, J. Seshu, and M. E. Konkel.** 2001. Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. J Infect Dis **183:**1607-1616.

122. **Robinson, D. A.** 1981. Infective dose of *Campylobacter jejuni* in milk. Br M J **282**.

123. **Ruiz, J., F. Goni, F. Marco, F. Gallardo, D. Mirelis, D. A. Jimenez, and J. Vila.** 1998. Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyrA* gene mutations in quinolone-resistant clinical isolates. Microbiol Immunol **42:**223-226.

124. **Ruiz-Palacios, G. M., J. Torres, N. I. Torres, E. Escamilla, B. R. Ruiz-Palacios, and J. Tamayo.** 1983. Cholera-like enterotoxin produced by *Campylobacter jejuni*. Characterisation and clinical significance. Lancet **2:**250-253.

125. **Russell, R. G., and D. C. Blake, Jr.** 1994. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. Infect Immun **62:**3773-3779.

126. **Sagara, H., A. Mochizuki, N. Okamura, and R. Nakaya.** 1987. Antimicrobial resistance of *Campylobacter jejuni* and Campylobacter coli with special reference to plasmid profiles of Japanese clinical isolates. Antimicrob Agents Chemother **31:**713-719.

127. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

128. **Sansonetti, P. J.** 1998. Molecular and cellular mechanisms of invasion of the intestinal barrier by enteric pathogens. The paradigm of *Shigella*. Folia Microbiol (Praha) **43:**239-246.

129. **Sansonetti, P. J., and C. Egile.** 1998. Molecular bases of epithelial cell invasion by *Shigella flexneri*. Antonie Van Leeuwenhoek **74:**191-197.

130. **Scheme, C. S. S.** 2002. Ciprofloxacin resistance in *Campylobacter jejuni*: case-case analysis as a tool for elucidating risks at home and abroad. J Antimicrob Chemother **50:**561-568.

131. **Sherburne, R., T. D. Lawley, M. W. Gilmour, F. R. Blattner, V. Burland, E. Grotbeck, D. J. Rose, and D. E. Taylor.** 2000. The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. Nucleic Acids Res **28:**2177-2186.

132. **Simon, R., U. Preifer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1:**784-790.

133. **Skirrow, M. B., and M. J. Blaser.** 2000. Clinical aspects of *Campylobacter* infection. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington.

134. **Smith, K., J. B. Bender, and M. T. Osterholm.** 2000. Antimicrobial resistance in animals and relevance to human infections. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.

135. **Smith, K. E., J. M. Besser, C. W. Hedberg, F. T. Leano, J. B. Bender, J. H. Wicklund, B. P. Johnson, K. A. Moore, and M. T. Osterholm.** 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. Investigation Team. N Engl J Med **340**:1525-1532.
136. **Sougakoff, W., B. Papadopoulou, P. Nordmann, and P. Courvalin.** 1987. Nucleotide sequence and distribution of gene *tetO* encoding tetracycline resistance in *Campylobacter coli*. FEMS Microbiol Lett **44**.
137. **Strack, B., M. Lessl, R. Calendar, and E. Lanka.** 1992. A common sequence motif, -E-G-Y-A-T-A-, identified within the primase domains of plasmid-encoded I- and P-type DNA primases and the alpha protein of the *Escherichia coli* satellite phage P4. J Biol Chem **267**:13062-13072.
138. **Szymanski, C. M., M. King, M. Haardt, and G. D. Armstrong.** 1995. *Campylobacter jejuni* motility and invasion of Caco-2 cells. Infect Immun **63**:4295-4300.
139. **Takata, T., T. Ando, D. Israel, T. M. Wassenaar, and M. J. Blaser.** 2003. A *dprA* homolouge, Cj0634, is involved in transformation of *Campylobacter jejuni*. J Bacteriol **Submitted**.
140. **Takata, T., S. Fujimoto, and K. Amako.** 1992. Isolation of nonchemotactic mutants of *Campylobacter jejuni* and their colonization of the mouse intestinal tract. Infect Immun **60**.
141. **Tauxe, R. V.** 2002. Emerging foodborne pathogens. Int J Food Microbiol **78**:31-41.
142. **Taylor, D. E.** 1992. Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* to tetracycline, chloramphenicol and erythromycin. *In* I. Nachamkin (ed.), *Campylobacter jejuni*: Current status and future trends. ASM Press, Washington, D.C.
143. **Taylor, D. E.** 1986. Plasmid-mediated tetracycline resistance in *Campylobacter jejuni*: expression in *Escherichia coli* and identification of homology with streptococcal class M determinant. J Bacteriol **165**:1037-1039.
144. **Taylor, D. E., and J. H. Bryner.** 1984. Plasmid content and pathogenicity of *Campylobacter jejuni* and *Campylobacter coli* strains in the pregnant guinea pig model. Am J Vet Res **45**:2201-2202.
145. **Taylor, D. E., N. Chang, R. S. Garner, R. Sherburne, and L. Mueller.** 1986. Incidence of antibiotic resistance and characterization of plasmids in *Campylobacter jejuni* strains isolated from clinical sources in Alberta, Canada. Can J Microbiol **32**:28-32.
146. **Taylor, D. E., S. A. De Grandis, M. A. Karmali, and P. C. Fleming.** 1981. Transmissible plasmids from *Campylobacter jejuni*. Antimicrob Agents Chemother **19**:831-835.
147. **Taylor, D. E., S. A. DeGrandis, M. A. Karmali, and P. C. Fleming.** 1980. Transmissible tetracycline resistance in *Campylobacter jejuni*. Lancet **2**:797.

148. **Taylor, D. E., K. Hiratsuka, H. Ray, and E. K. Manavathu.** 1987. Characterization and expression of a cloned tetracycline resistance determinant from *Campylobacter jejuni* plasmid pUA466. J Bacteriol **169:**2984-2989.

149. **Taylor, D. E., L. J. Jerome, J. Grewal, and N. Chang.** 1995. Tet(O), a protein that mediates ribosomal protection to tetracycline, binds, and hydrolyzes GTP. Can J Microbiol **41:**965-970.

150. **Taylor, D. E., D. G. Newell, and A. D. Pearson.** 1983. Incidence of plasmid DNA in strains of *Campylobacter jejuni* isolated from stool specimens at 37 C and 43 C. J Infect Dis **147:**965-966.

151. **Taylor, D. E., L. K. Ng, and H. Lior.** 1985. Susceptibility of *Campylobacter* species to nalidixic acid, enoxacin, and other DNA gyrase inhibitors. Antimicrob Agents Chemother **28:**708-710.

152. **Tee, W., A. Mijch, E. Wright, and A. Yung.** 1995. Emergence of multidrug resistance in *Campylobacter jejuni* isolates from three patients infected with human immunodeficiency virus. Clin Infect Dis **21:**634-638.

153. **Tenover, F. C., T. Gilbert, and P. O'Hara.** 1989. Nucleotide sequence of a novel kanamycin resistance gene, *aphA-7*, from *Campylobacter jejuni* and comparison to other kanamycin phosphotransferase genes. Plasmid **22:**52-58.

154. **Tenover, F. C., S. Williams, K. P. Gordon, C. Nolan, and J. J. Plorde.** 1985. Survey of plasmids and resistance factors in *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob Agents Chemother **27:**37-41.

155. **Tobe, T., T. Hayashi, C.-G. Han, G. K. Schoolnik, E. Ohtsubo, and C. Saskawa.** 1999. Complete DNA sequence and structural analysis of the enteropathogenic *Escherichia coli* adherence factor plasmid. Infect Immun **67:**5455-5462.

156. **Travers, K., and M. Barza.** 2002. Morbidity of infections caused by antimicrobial-resistant bacteria. Clin Infect Dis **34 Suppl 3:**S131-S134.

157. **Trieber, C. A., N. Burkhardt, K. H. Nierhaus, and D. E. Taylor.** 1998. Ribosomal protection from tetracycline mediated by Tet(O): Tet(O) interaction with ribosomes is GTP-dependent. Biol Chem **379:**847-855.

158. **Trieber, C. A., and D. E. Taylor.** 2000. Mechanisms of antibiotic resistance in *Campylobacter*. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.

159. **Unit, B.-G.-O. S. H.** 2000. The investigative report of the Walkerton outbreak of waterborne gastroenteritis. Bruce-Grey-Owen Sound Health Unit.

160. **Uyttendaele, M., R. Schukkink, B. Van Gemen, and J. Debevere.** 1999. Incidence of Salmonella, *Campylobacter jejuni*, *Campylobacter coli*, and *Listeria monocytogenes* in poultry carcasses and different types of poultry products for sale on the Belgian retail market. J Food Prot **62:**735-740.

161. **van der Bogaard, A. E., N. London, C. Driessen, and E. E. Stobberingh.** 2001. Antibiotic resistance of *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J Antimicrob Chemother **47:**763-771.

162. **van Vliet, A. H., and J. M. Ketley.** 2001. Pathogenesis of enteric *Campylobacter* infection. Symp Ser Soc Appl Microbiol:45S-56S.

163. **van Vliet, A. H., A. C. Wood, J. Henderson, K. Woolridge, and J. M. Ketley.** 1998. Genetic manipulation of enteric *Campylobacter* species, Methods in Microbiology, vol. 27. Academic Press Ltd.

164. **Vazquez-Torres, A., and F. C. Fang.** 2000. Cellular routes of invasion by enteropathogens. Curr Opin Microbiol **3.**

165. **Villedieu, A., M. L. Diaz-Torres, N. Hunt, R. McNab, D. A. Spratt, M. Wilson, and P. Mullany.** 2003. Prevalence of tetracycline resistance genes in oral bacteria. Antimicrob Agents Chemother **47:**878-882.

166. **Wallace, J. S., K. N. Stanley, J. E. Currie, P. J. Diggle, and K. Jones.** 1997. Seasonality of thermophilic *Campylobacter* populations in chickens. J Appl Micro **82:**219-224.

167. **Wang, Y., W. M. Huang, and D. E. Taylor.** 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. Antimicrob Agents Chemother **37:**457-463.

168. **Wang, Y., and D. E. Taylor.** 1990. Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression, and cloning vector construction. Gene **94:**23-28.

169. **Wang, Y., and D. E. Taylor.** 1991. A DNA sequence upstream of the *tet*(*O*) gene is required for full expression of tetracycline resistance. Antimicrob Agents Chemother **35:**2020-2025.

170. **Wang, Y., and D. E. Taylor.** 1990. Natural transformation in *Campylobacter* species. J Bacteriol **172:**949-955.

171. **Wassenaar, T. M., B. N. Fry, and B. A. van der Zeijst.** 1993. Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electro-transformation. Gene **132.**

172. **Wassenaar, T. M., B. A. van der Zeijst, R. Ayling, and D. G. Newell.** 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. J Gen Microbiol **139:**1171-1175.

173. **White, D. G., S. Zhao, S. Simjee, D. D. Wagner, and P. F. McDermott.** 2002. Antimicrobial resistance of foodborne pathogens. Microb Infect **4:**405-412.

174. **Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett.** 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. Infect Immun **66:**1934-1940.

175. **WHO.** 2000. Global principles for the containment of antimicrobial resistance due to antimicrobial use in animals intended for food. World Health Organization.

176. **Wistrom, J., and S. R. Norrby.** 1995. Fluoroquinolones and bacterial enteritis, when and for whom? J Antimicrob Chemother **36:**23-39.

177. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. Science **279:**996-997.

178. **Yan, W., and D. E. Taylor.** 1991. Characterization of erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli.* Antimicrob Agents Chemother **35:**1989-1996.

179. **Yao, R., R. A. Alm, T. J. Trust, and P. Guerry.** 1993. Construction of new *Campylobacter* cloning vectors and a new mutational cat cassette. Gene **130:**127-30.

180. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni* defective in invasion of eukaryotic cells: the role of flagella in invasion. Mol Microbiol **14.**

181. **Yao, R., D. H. Burr, and P. Guerry.** 1997. CheY-mediated modulation of *Campylobacter jejuni* virulence. Mol Microbiol **23:**1021-1031.

182. **Zhang, S., R. A. Kingsley, R. L. Santos, H. Andrews-Polymenis, M. Raffatellu, J. Figueriedo, J. Nunes, R. M. Tsolis, L. G. Adams, and A. J. Aumler.** 2003. Molecular pathogenesis of *Salmonella enterica* serotype Typhimirium-induced diarrhea. Infect Immun **71:**1-12.

183. **Ziprin, R. L., C. R. Young, L. H. Stanker, M. E. Hume, and M. E. Konkel.** 1999. The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. Avian Dis **43:**586-589.

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Re: Permission to use figure from an article in Infection and Immunity
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Good day,

I am writing to request the use of a figure published in Infection and Immunity for my Master of Science thesis at the University of Alberta.

The figure I wish to use is from the article by **Bacon *et al.*, "Involvement of a Plasmid in Virulence of *Campylobacter jejuni* 81-176" *Infection and Immunity* 68(8):4384-4390.**

The figure is "Figure 1: *Bgl*II digestion pattern of plasmids isolated from *C. jejuni*." For clarity, I have attached a copy of the figure in question.

Unfortunately I have to submit my Masters thesis within the next week, and I would be grateful if you could respond by June 11, 2003.

I can be contacted via email at

Thank you for your time,

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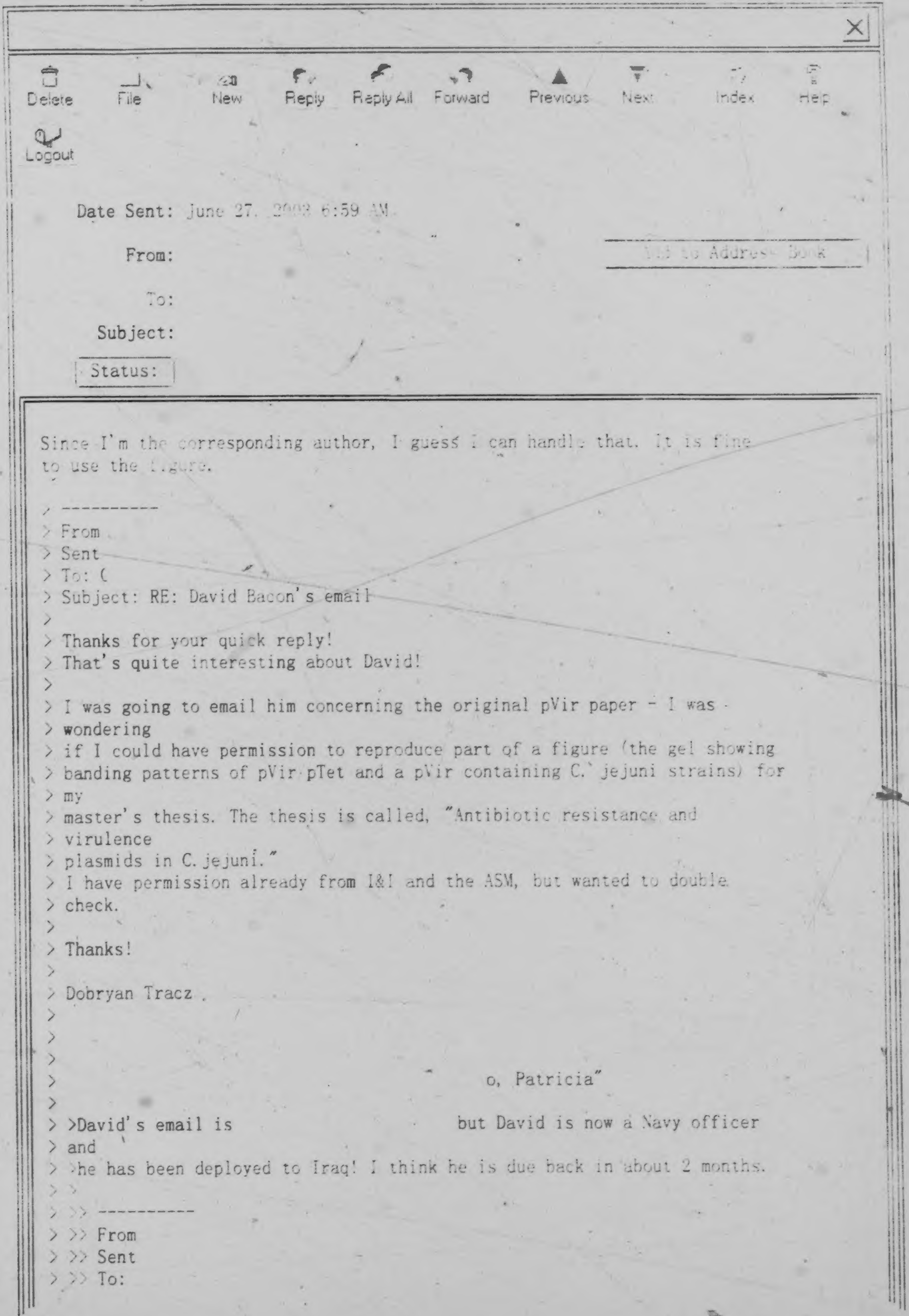
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